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Targeting IL-23/T_H17 Axis in Suppressing Intestinal Inflammation

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Abstract

IL-23/T_H17 inflammatory responses are regarded as central to the pathogenesis of inflammatory bowel disease (IBD). Human genome wide association studies demonstrate that *IL23R* gene polymorphisms are associated with susceptibility to IBD. T_H17 cells and the IL-17A cytokine are highly produced in the intestinal mucosa of patients with IBD. However, in clinical trials anti-IL-17A antibodies have shown low efficacy and increased infections in Crohn's disease. Hence the role of the IL-23/T_H17 axis in experimental colitis models and human IBD needs further examination.

In order to study the IL-23/T_H17 axis, a murine spontaneous colitis model, *Winnie* mice, were used in the study. *Winnie* mice have a single missense mutation in the *Muc2* mucin gene which results in misfolding of the mucin during biosynthesis, epithelial secretory cell endoplasmic reticulum stress, and mucosal inflammation. Thus in this model the defect arises in the epithelium, with the inflammation driven by the response from a competent immune system. *Winnie* mice share similarities with human ulcerative colitis, developing progressive intestinal inflammation and driven by a mixed but IL-23/T_H17-dominated proinflammatory response.

In a transfer colitis model, IL-17A$^{-/-}$ or IL-17Ra$^{-/-}$ naïve T cells were transferred into immunodeficient recipient *Rag1*$^{-/-}$ mice. *Rag1*$^{-/-}$ recipients with an epithelial defect arising from the *Winnie* allele were also included to examine the contribution of a defect in intestinal epithelial integrity to the development of colitis. Results from transfer experiments demonstrated thatIL-17A production by the donor T cell is not necessary to induce transfer colitis, and IL-17Ra-deficient T cells were more colitogenic than wild type T cells, suggesting that IL-17Ra signaling in T cells plays a protective role in the transfer model of colitis. To further investigate the physiological role of IL-17A in intestinal inflammation, *Il17a*$^{-/-}$*/Winnie* mice were generated by crossing *Il17a*$^{-/-}$ mice with *Winnie* mice. *Il17a*$^{-/-}$*/Winnie* mice at 8 weeks of age had similar histological colitis compared to *Winnie*. Interestingly, colitis severity did not progress with age in *Il17a*$^{-/-}$*/Winnie* mice. Therefore, IL-17A is involved in the progression rather than the initiation of *Winnie* colitis.

Next, in an attempt to mimic biological therapy with antibodies, the efficacy of anti-IL-17A antibodies in *Winnie* with emerging and established colitis was assessed. Neutralizing IL-17A in *Winnie* mice with emerging colitis failed to alleviate colonic inflammation. Blocking IL-17A or IL-17Ra in *Winnie* mice with established colitis resulted in

similar severity of histological colitis compared to isotype treated mice, suggesting targeting the IL-17A-IL-17Ra axis using monoclonal antibodies is not capable of suppressing intestinal inflammation in the *Winnie* colitis model.

In contrast, neutralizing IL-23 using an anti-p19 antibody significantly alleviated emerging colitis, downregulating *Il17a* and *Il1b* gene expression. Similarly, two weeks treatment with anti-p19 antibody significantly ameliorated established colitis compared to *Winnie* mice receiving isotype antibody. Anti-p19 decreased colon weight and histological colitis scores and was superior to anti-p40 antibody. In contrast to *Winnie* mice with emerging colitis, anti-p19 treatment of mice with established colitis did not modify proinflammatory cytokines. However, anti-p19 diminished colonic neutrophil infiltration, suggesting that the efficacy of anti-IL-23 treatment in *Winnie* established colitis may be due to mechanisms other than direct suppression of cytokine-producing immune cells. Anti-p19 downregulated *Mip2a* chemokine mRNA expression in isolated colonic epithelial cells and restored colonic goblet cell mucin production.

IL-23 has previously been thought to act only on leukocytes, however, my experiments show that IL-23 acts directly on intestinal epithelial cells. Treatment of human colonic LS174T cells with recombinant IL-23 confirmed my *in vivo* findings. IL-23 treatment increased intracellular reactive oxygen species (ROS) levels in a Stat3 transcription factor-dependent manner in this human colonic cell line. The IL-23-driven ROS decreased MUC2 mucin production, and increased release of the chemokine, IL-8. Due to these multiple mechanisms of action, neutralization of IL-23 in colitis is likely to be efficacious not only via effects on immune cells, but by reducing chemokine-mediated recruitment of inflammatory cells into the intestine, and by replenishing intestinal mucus, thereby helping restore normal intestinal homeostasis.

In summary, results from this thesis show that blocking T_H17-associated effector cytokines or cytokine receptor is not effective in suppressing intestinal inflammation. However, targeting IL-23 alone is sufficient to suppress colitis in *Winnie* mice. The previously unrecognised adverse effects of IL-23 on colonic epithelial cells may partially underlie the protection from IBD conveyed by hypomorphic IL-23 receptor polymorphisms, and contribute to the efficacy of IL-23 neutralizing antibodies in IBD.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Publications during candidature

Peer-reviewed papers

Wang R, Hasnain SZ, Tong H, Das I, Oancea I, Chen AC, Proctor M, Florin TH, Eri R, McGuckin MA. Neutralizing IL-23 is superior to blocking IL-17 in suppressing intestinal inflammation in a murine model of spontaneous colitis. *Inflammatory Bowel Diseases* (In press)

Hasnain SZ, Borg D, Harcourt B, Tong H, Sheng YH, Ng CP, Das I, **Wang R**, Chen A, Loudovaris T, Kay TW, Thomas HE, Forbes J, Whitehead J, Prins JB and McGuckin MA. Glycemic control in diabetes is restored by therapeutic manipulation of cytokines that regulate beta cell stress. *Nature Medicine* 20: 1417-1426

Sheng YH, Triyana S, **Wang R**, Das I, Gerloff K, Florin TH, Sutton P, McGuckin MA. 2013. MUC1 and MUC13 differentially regulate epithelial inflammation in response to inflammatory and infectious stimuli. *Mucosal Immunol* 6: 557-68

Benham H, Rehaume LM, Hasnain SZ, Velasco J, Baillet AC, Ruutu M, Kikly K, **Wang R**, Tseng H-W, Thomas GP, Brown MA, Strutton G, McGuckin MA, Thomas R. 2014. IL-23-mediates the intestinal response to microbial beta-glucan and the development of spondyloarthritis pathology in SKG mice. *Arthritis Rheumatol* 66:1755-1767

Manuscripts in preparation

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Contributions by others to the thesis

All work was carried out by Ran Wang, except for the following:

Ms Wendy Tong performed the *IL-17A*$^{-/-}$ T cell transfer experiment.

The Ly6G/6C immunohistochemistry was carried out by Dr Clay Winterford.

All the paraffin tissue embedding and cutting of histological sections were done by either Dr Clay Winterford's laboratory at Queensland Institute of Medical Research or Ms Crystal Chang at Translational Research Institute.

Dr. Sumaira Hasnain performed the ERAI transfection assay in LS174T cells and the MUC2 ELISA.

Dr. Yonghua Sheng helped with the immunoblotting analyses.

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Inflammatory bowel diseases, colitis, cytokines, IL-17A, IL-23, epithelial cells, ER stress, oxidative stress, neutrophil

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List of Abbreviations

5-ASA	5-aminosalicylic acid
6-MP	6-mercaptopurine
Agr2	anterior gradient 2
AhR	aryl hydrocarbon receptor
APCs	antigen presenting cells
APRIL	a proliferation-inducing ligand
ATG16L1	autophagy-related protein 16-1
AZA	azathioprine
BAFF	B cell-activating factor
CARD9	caspase-associated recruitment domain 9
CCR6	C-C chemokine receptor type 6
CD	Crohn's disease
DCs	dendritic cells
DHE	dihydroethidium
DMSO	dimethyl sulphoxide
DSS	dextran sodium sulphate
ER	endoplasmic reticulum
Foxp3	forkhead box P3
GM-CSF	granulocyte-macrophage colony stimulating factor
GSH	glutathione
GWAS	genome-wide association studies
IELs	intraepithelial lymphocytes
IKK	IκB kinase
IL	interleukin
ILCs	innate lymphoid cells
JAK2	Janus kinase 2
LPS	lipopolysaccharide

MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
mLNs	mesenteric lymph nodes
MMPs	matrix metalloproteinases
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NOD2	nucleotide-binding oligomerization domain-containing protein 2
PAS	periodic acid Schiff's (PAS)
RA	retinoic acid
Rag	recombination-activating genes
ROR	retinoic acid receptor related orphan receptor
ROS	reactive oxygen species
SCID	severe combined immunodeficiency
SNP	single-nucleotide polymorphism
SOD	superoxide dismutases
STAT	signal transducer and activator of transcription
TGF-β	transforming growth factor-β
TLR	toll like receptor
Tm	tunicamycin
TNBS	trinitrobenzene sulfonic
TNF-α	tumor necrosis factor alpha
TSLP	thymic stromal lymphopoietin
UC	ulcerative colitis
UPR	unfolded protein response
Xbp1	X-box binding protein 1

1.0 Literature Review

1.1. The intestinal immune system

The intestinal mucosa is the preferred niche of an enormous number of commensal bacteria (up to 10^{14}) (Macpherson and Harris, 2004), and also requires selective permeability for nutrient absorption. Prevention of bacterial penetration into the submucosa and systemic spread is important to maintain mucosal homeostasis. More than 50% of the human body's lymphocytes are resident in the mucosa of the intestine, and the intestinal mucosa harbours the largest number of tissue macrophages of any organ (Macpherson and Uhr, 2004a). Therefore, the intestinal immune system must be able to strike a balance between mounting an effective immune response against external pathogens and maintaining tolerance to commensal luminal microbes. Interactions between the highly adapted epithelial barrier and the underlying innate/adaptive immune system play a central role in this process.

1.1.1 The epithelial barrier in maintaining intestinal homeostasis

The epithelial barrier of the intestinal tract comprises a monolayer of epithelial cells, including enterocytes, enteroendocrine cells, goblet cells, Paneth cells and crypt-resident stem cells, which are interconnected by tight-junctions. The barrier is constantly renewed and regenerated by intestinal epithelial stem cells located at the base of the crypts. Enterocytes are the major cell population in the intestinal epithelium, which has an absorptive phenotype adapted for metabolic and digestive functions. Enteroendocrine cells secrete hormones and regulate digestive functions of the intestine. Other secretory epithelial cells including goblet cells and Paneth cells are specialized in maintaining barrier functions of the intestinal epithelium.

The secreted mucus barrier, a viscous, gel-like film, serves as the first physical and chemical barrier to prevent direct contact of luminal contents with epithelial cells. Luminal microbes can only transverse into the outer mucus layer under normal physiological conditions, while the inner layer is sterile and filled with high concentrations of antimicrobial molecules (Johansson et al., 2008; McGuckin et al., 2009). Mucins, the major macromolecular components of the mucus layer, are highly glycosylated proteins usually secreted by specialized secretory cells known as goblet cells. Antimicrobial molecules, the other important component of mucus layer, in the small intestine are mainly produced by

Paneth cells, which are highly specialized epithelial secretory cells present at the base of crypts (Ayabe et al., 2000; Wilson et al., 1999). Paneth cells are not present in the non-inflamed colon where other enterocytes produce anti-microbial molecules. Upon exposure to microbes, Paneth cells can secret α-defensins (cryptidins in mice) and other antimicrobial molecules such as lysozyme to stop bacterial penetration into intestinal mucosa (Ouellette, 2010).

Besides producing the secreted mucus barrier, epithelial cells are also actively involved in the process of defence against bacterial infiltration (Peterson and Artis, 2014). Intestinal epithelial cells internalize Gram-negative bacteria through Toll-like receptor (TLR)-4-dependent phagocytosis (Neal et al., 2006). Peyer's patches in the small intestine are covered by follicle-associated epithelium lacking goblet cells and a secreted mucus barrier. Microfold cells (M cells) in this specialized epithelium allow uptake of luminal bacteria and a variety of antigens for presentation to underlying antigen presenting cells. Until recently, antigen transportation through the epithelium was thought to be a unique feature of M cells, however it has now been shown that small intestinal goblet cells can deliver luminal soluble antigens to underlying $CD103^+$ dendritic cells which further induce IgA production and promote lamina propria regulatory T cell development (Knoop et al., 2014; McDole et al., 2012). After epithelial cell phagocytosis, antigens are translocated to antigen presenting cells which migrate to the mesenteric lymph nodes, stimulating lymphocytes and inducing a non-inflammatory immune response characterized by IgA production by B cells (Hathaway and Kraehenbuhl, 2000; Macpherson and Uhr, 2004b).

Intestinal epithelial cells are able to alter their phenotype and produce an array of proinflammatory chemokines, cytokines and antimicrobial defence molecules when stimulated by pathogenic luminal microbes or proinflammatory stimuli derived from cells in the intestinal mucosa (Kagnoff, 2014). It has been shown that probiotic *Lactobacillus acidophilus NCFM* induces IL-1α and lL-1β secretion via the NF-κB p65 and p38 mitogen-activated protein kinase (MAPK) pathways in intestinal epithelial cells (Jiang et al., 2012). Metabolic products of the commensal flora including phytic acid (Wawszczyk et al., 2012) and butyrate (Kim et al., 2013b) play an important role in regulating IL-8 secretion from colonic epithelial cells via the MAPK pathway. In summary, intestinal epithelial enterocytes and secretory cells are more than a passive barrier; they act like innate immune cells and

are actively involved in regulating microbial antigen presentation to the immune system and maintaining intestinal immune homeostasis (Figure.1.1).

Evidence from murine models demonstrates the importance of epithelial integrity and function for maintaining intestinal homeostasis. Epithelial-restricted gene mutations or deficiencies can lead to spontaneous colitis and result in inflammatory bowel disease-like phenotypes in the mouse colon (Heazlewood et al., 2008; Kaser et al., 2008; Nenci et al., 2007; Park et al., 2009; Van der Sluis et al., 2006; Wei et al., 2012).

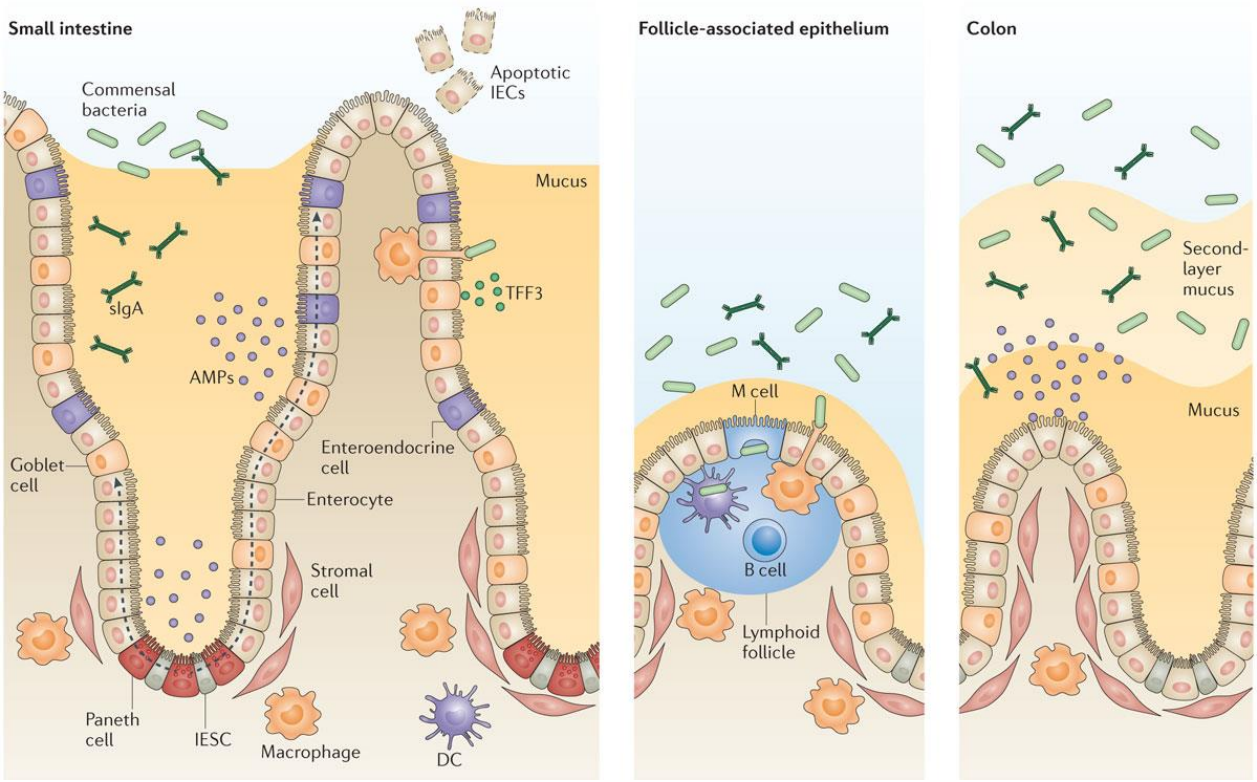

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Figure 1.1 The intestinal epithelial barrier is actively involved in maintaining local mucosal immune homeostasis. Most of bacteria reside in the faecal material in the lumen or in the outer layer of mucus which covers the intestinal epithelial cells. Intestinal epithelial cells (IECs) form a biological and physical barrier that separates luminal microbes from the mucosal immune system. The intestinal epithelial cells include enterocytes, enteroendocrine cells, goblet cells, Paneth cells and crypt-resident stem cells which control the continuous regeneration of the epithelial cell layer. Differentiated IECs, with the exception of Paneth cells, migrate up the crypt–villus axis. Secretory goblet cells and Paneth cells secrete mucus and antimicrobial proteins (AMPs) to keep the inner mucus layer sterile and promote the exclusion of bacteria from the epithelial surface. The release of secretory IgA (sIgA) further contributes to this barrier function. Microfold cells (M cells) and goblet cells are constantly sampling and mediating the transport of luminal antigens for presentation to antigen presenting cells. Occasionally, commensal microbes penetrate through the mucus layer and reach epithelial cells. Lamina propria resident macrophages are able to kill penetrating bacteria by phagocytosis, while some may live in DCs for several days after phagocytosis. This allows DCs to interact with B and T cells within the inter-follicular area and induce IgA production by B cells and non-inflammatory cytokine secretion by T lymphocytes (Peterson and Artis, 2014)(Copyright obtained from Nature Publishing Group).

1.1.2 Innate immune defence system of the intestinal tract

Under physiological conditions, commensal microbes occasionally reach the epithelium. However, lamina propria innate immune cells are usually able to phagocytose these microbes, preventing further penetration whilst maintaining local tolerance. Unlike professional antigen presenting cells residing in other tissues, intestinal macrophages and dendritic cells (DCs) do not activate naïve T cells to induce inflammatory responses in response to TLR activation. Furthermore, both macrophages and DCs contribute to maintenance of intestinal homeostasis by induction of tissue resident T_{reg} maturation (Coombes and Maloy, 2007; Denning et al., 2011; Hadis et al., 2011).

Intestinal tissue macrophages are closely regulated by the luminal microflora. During the maturation of the intestinal immune system, macrophages down-regulate expression of co-stimulatory factors such as CD40, CD80 and CD86. Lamina propria resident macrophages do not express innate response receptors to lipopolysaccharide (LPS), IgA, IgG, growth factor receptors for IL-2 and IL-3 or toll like receptor 4 (TLR4) (Smythies et al., 2005). As a result, they are unable to respond to LPS and other microbe-associated molecules derived from the luminal flora (Smith et al., 2001). Intestinal macrophages also greatly reduce production of proinflammatory cytokines in response to inflammatory stimuli, including IL-1β, IL-6, IL-12, TGF-β and TNF-α (Fadok et al., 1998). However, their strong phagocytic ability remains intact (Smith et al., 2005; Smythies et al., 2005). Apoptotic cells or bacterial antigens taken up by macrophages can also induce T cell tolerance as a result of presentation in the absence of costimulatory factors (Hershberg and Mayer, 2000). Furthermore, a recent study demonstrates that IL-23 producing intestinal $CX3CR1^{+}$ mononuclear phagocytes induce IL-22 production in group 3 innate lymphoid cells, which offers protection in a *Citrobacter rodentium*-induced experimental colitis model (Longman et al., 2014).

Intestinal DCs are key players in mediating interactions between the epithelial barrier and adaptive immune cells. DCs are derived from circulating monocytes and migrate to the lamina propria, following chemokine-gradients produced by epithelial cells (Cook et al., 2000). Under healthy conditions, $CD103^{+}$ DCs take up antigens from luminal commensal flora, and then migrate to mesenteric lymph nodes where they induce IgA production by B cells, and T cell tolerance (Huang et al., 2000; Schulz et al., 2009).

Internalized bacteria can survive several days within DCs restricted to the mucosal immune system, and this interaction is believed to help selectively induce IgA secretion, which helps prevent bacterial penetration of mucus (Macpherson and Harris, 2004). DCs present in Peyer's patches under the M-cells of the dome epithelium are able to induce $Foxp3^+$ regulatory T cell differentiation via a TGF-β and retinoic acid-dependent mechanism (Coombes and Maloy, 2007; Schulz et al., 2009; Sun et al., 2007).

Innate lymphoid cells (ILCs) are a recently defined cell population playing an important role in intestinal homeostasis. ILCs have been found at multiple mucosal sites and barrier surfaces of the mouse and human, including lung (Monticelli et al., 2012), skin (Kim et al., 2013a) and intestine (Tait Wojno and Artis, 2012). They are actively involved in tissue remodelling, local inflammatory responses and early innate immune responses to infection. ILCs have been characterized according to their differing transcription factor and cytokine profiles into group 1, group 2 and group 3, which share similarities with the $CD4^+$ T_H1, T_H2 and T_H17 cells, respectively (Walker et al., 2013). Group 1 ILCs, including the natural killer (NK) cells and innate lymphoid cell subset 1 (ILC1), produce the T_H1-associated cytokines IFN-γ and TNFα in response to IL-12 and IL-15 (Fuchs et al., 2013). In an important difference to NK cells, ILC1s are unable to produce perforin and granzyme B and therefore lack direct cytotoxic ability. The actual physiological role of ILC1s is still unclear, although a study reported that the frequency of ILC1s in the inflamed intestinal mucosa of Crohn's disease patients was substantial increased (Bernink et al., 2013). Group 2 ILCs (ILC2) produce the T_H2-associated cytokines IL-5 and IL-13. ILC2s have been shown play a protective role in early innate immune responses against intestinal helminth infection by promoting goblet cell hyperplasia and stimulating mucin secretion (Neill et al., 2010). Group 3 ILCs are driven by the transcription factor $ROR\gamma t^+$, and produce the T_H17 cytokines IL-17 and IL-22 in response to IL-23 (Bernink et al., 2013). IL-22 produced by ILC3s has a protective role in stimulating intestinal epithelial regeneration following injury or bacterial infection (Sonnenberg et al., 2011; Zenewicz et al., 2008). ILC3-derived IL-22 has also been shown to regulate selective containment of lymphoid-resident *Alcaligenes* to prevent systemic inflammation associated with chronic infections (Sonnenberg et al., 2012). ILC3-derived IL-17A is shown to have proinflammatory effects in murine colitis models as well as human inflammatory bowel diseases (IBD) (Buonocore et al., 2010a; Coccia et al., 2012; Geremia et al., 2011). Although the importance of ILCs in intestinal homeostasis has been appreciated in various murine models, much of the

research has been conducted in models lacking lymphocytes, and the contribution of ILCs to human disease pathogenesis needs further investigation.

To regulate immune responses against luminal flora and maintain intestinal homeostasis, innate immune cells closely interact with intestinal epithelial cells. This cross-talk is a two-way communication. Lamina propria DCs and intestinal resident macrophages are able to extend dendrites through epithelial tight junctions to sample luminal bacteria antigens, while the integrity of the epithelial layer is preserved as DCs also express tight-junction proteins (Rescigno et al., 2001). On the other hand, epithelial cells produce thymic stromal lymphopoietin (TSLP), TGFβ and retinoic acid (RA) which induce conditioned non-inflammatory DCs to further stimulate homeostatic T_H2 and T_{reg} immune responses (Peterson and Artis, 2014). Intestinal epithelial cells also play a crucial part in regulating ILCs in response to commensal bacteria and antigens. RORγt$^+$ ILC3s are responsible for the majority of intestinal IL-22 production in health, especially in absence of adaptive immunity, and this is downregulated by IL-25 produced by epithelial cells in response to symbiotic microbes (Sawa et al., 2011). Intestinal epithelial cells are also able to induce T_H2 immunity during helminth infection. Epithelial cell-derived TSLP and IL-25 stimulate the differentiation of haematopoietic progenitor cells towards myeloid progenitor and basophil progenitor phenotypes which produce type 2 cytokines and promote T_H2 immune responses at the mucosal site to aid worm expulsion (Saenz et al., 2013; Saenz et al., 2010; Siracusa et al., 2013) (Figure 1.2 Innate immune regulation).

1.1.3 The adaptive immune system of gastrointestinal tract

Intestinal B cells and differentiated T cells, together with epithelial secretory cells provide the last line of defence to luminal bacteria. Following antigen uptake by antigen presenting cells (APCs), mucosal B cells produce IgA (Kallies et al., 2004) and T cells produce IL-4 (Rimoldi et al., 2005), both of which boost the barrier to microbes in a non-inflammatory way. Increased regulatory T cells in the intestinal mucosal system are another feature of mucosal adaptive immunity. The gut microenvironment and local cytokine milieu favors TCR activated CD4$^+$ T cell differentiation into IL-10 and TGF-β producing cells with low proliferation ability (Dubois et al., 2005). The generation of a T_H2/T_{reg} dominant regulatory environment is modulated by commensal flora and restricted to the mucosal immune system. Following stimulation in Peyer's patches, activated B and T cells re-enter the bloodstream through intestinal lymphatics and then circulate back to

the intestinal mucosa (Figure 1.2). A specialized T cell population, intraepithelial lymphocytes (IELs), is present in the intestinal mucosa which has close interactions with epithelial cells in maintaining barrier functions (Satoh-Takayama et al., 2008). IELs including γδ T cells and NKT cells (Cheroutre et al., 2011; Ismail et al., 2011). Although the exact local environment in promoting IELs development is unknown, the intimate crosstalk between IELs and epithelial cells suggest that epithelial cells play an important role in regulating their differentiation and function.

Epithelial cells also regulate the maturation process of naïve B cells to IgA-secreting plasma cells in the lamina propria. In response to commensal bacteria-induced NF-κB signalling, intestinal epithelial cells produce proliferation-inducing ligand (APRIL) and B cell-activating factor (BAFF) which directly stimulate heavy chain class-switch recombination of B cells (He et al., 2007; Xu et al., 2007). This process can be facilitated and amplified by epithelial cell-conditioned mucosal DCs and macrophages (Cerutti, 2008). Epithelial-derived TSLP induces APRIL and BAFF production by mucosal DCs, which further acts on B cells to promote class-switching and IgA production (He et al., 2007; Xu et al., 2007). Specialized intestinal epithelial cells, capable of translocating luminal antigens, also express major histocompatibility complex (MHC) class I and class II molecules suggesting that epithelial cells may act like APCs for lamina propria $CD4^+$ and $CD8^+$ T cells (Zimmer et al., 2000). Unlike the classical presentation which stimulates T cell activation, epithelial cells induce T cell anergy due to a lack of costimulatory factors (Hershberg and Mayer, 2000). Highly adapted NK cells and T cells also reside within the intestinal epithelial layer (Cheroutre, 2005). The close and active communication between the epithelial barrier and innate and adaptive immune cells establishes a complex and effective surveillance system to preserve intestinal homeostasis.

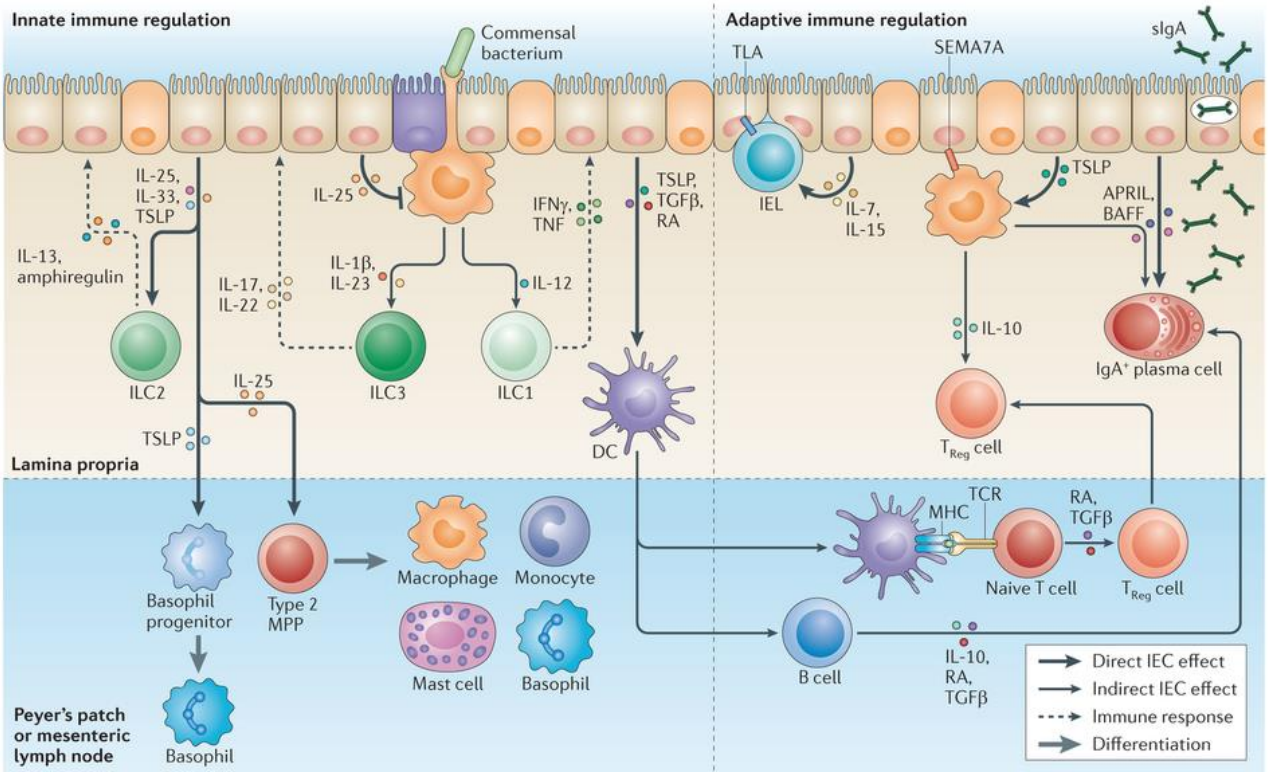

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Figure 1.2 The crosstalk between intestinal epithelial cells and underlying immune cells. Intestinal epithelial cell-derived cytokines IL-25, IL-33 and TSLP promote the expansion and differentiation of ILC2s, whereas IL-25 suppresses ILC1 and ILC3 function by limiting antigen presenting cell production of pro-inflammatory cytokines, especially IL-23. Epithelial cells prime migratory DCs and tissue resident macrophages towards a tolerogenic phenotype through the production of TSLP, TGFβ and retinoic acid. These DCs promote the differentiation of naive T cells into T_{reg} cells and the maturation of B cells into IgA-secreting plasma cells. After trafficking to the intestine, T_{reg} cells are expanded in number by macrophages that are conditioned to produce IL-10 by TSLP-mediated stimulation and through contact-dependent interactions with intestinal epithelial cells (Peterson and Artis, 2014)(Copyright obtained from Nature Publishing Group).

1.2. Dysregulated T cell responses and inflammatory cytokines in inflammatory bowel diseases

1.2.1 Inflammatory T cell responses in the intestinal immune system

Although mucosal immune responses are tightly regulated, inappropriate host immune responses to the commensal microflora can occur, inducing local inflammation and tissue destruction. Intestinal inflammation can result from multi-level triggers including

primary defects in the epithelial barrier, chronic or continuous bacterial infection or penetration, changes in the threshold of immune responses against luminal commensal microbes and a deviation in the type of immune response (McGuckin et al., 2009). These factors are not exclusive to each other, and combinations of triggers could result in a pathogenic inflammatory response involving both innate and adaptive immunity with increased proinflammatory cytokine production such as IL-1β, IFN-γ, IL-6, TNF-α, IL-17A and IL-23 (Figure. 1.3). IBD are chronic intestinal disorders of the gastrointestinal tract, and are typically classified as either Crohn's disease (CD) or ulcerative colitis (UC), although these can be difficult to distinguish and can be classified as indeterminate colitis. The complex aetiology of IBD is still not fully understood, but increased inflammatory cytokine production and dysregulated T cell responses are associated with the initiation and perpetuation of intestinal inflammation (Kaser et al., 2010; Troncone et al., 2013).

T_H1 and T_H2

T_H1 cells are induced by IL-12 and characterized as IFN-γ producing cells, whereas the signature cytokines of T_H2 cells are IL-4, IL-5 and IL-13. CD was believed to be a T_H1 mediated disease, and UC was thought to be driven by a T_H2 immune response. In patients with CD, disease pathology was linked with proinflammatory immune responses with greatly increased IL-12, IFN-γ and TNFα production, while high levels of IL-5 and IL-13 secretion were observed in patients with UC (Fuss et al., 1996; Monteleone et al., 1997). In addition, abundant production of IFN-γ from lamina propria lymphocytes was observed in patients with CD. High levels of STAT4 and T-bet, typical T_H1 associated transcription factors, were isolated from inflamed mucosal of patients with CD indicative T_H1 response may responsible for the progression of CD (Monteleone et al., 1997). An antibody against the IL-12 p40 unit proved effective in clinic trials in IBD supporting a pathogenic role of IL-12 (Mannon et al., 2004). However, with the discovery of IL-23 - a cytokine sharing the p40 subunit of IL-12 (Cua et al., 2003), the IL-23/T_H17 axis has been demonstrated to play a major role in the pathogenesis of IBD (Elson et al., 2007; Hue et al., 2006). More detailed cytokine biology will be discussed in the following sections.

T_H17

The physiological role of T_H17 cells is to prosecute immune responses against extracellular pathogens, particularly in mucosal tissues such as the respiratory and intestinal tracts, through a mechanism of secreting inflammatory cytokines, chemokines

and anti-microbial proteins (Bettelli et al., 2008; Khader et al., 2007; Ye et al., 2001). T_H17 cells are most abundant in the lamina propria of the small intestine, where their presence is boosted by the colonization of the intestinal segmented filamentous bacteria (SFB) (Atarashi et al., 2008; Ivanov et al., 2009; Lochner et al., 2011). While SFB are regarded as commensals by many and are present in neonatal life in mouse and man, they bear many of the features of pathogens, including mucus penetration, attachment to epithelium, activation of an inflammatory immune response and subsequent immune-mediated clearance. The mechanism of T_H17 induction by SFB has recently been revealed. SFB-encoded peptide antigens induce specific antigen receptor-bearing T cells differentiated into RORγt-expressing T_H17 cells, therefore, the effector function and antigen specificity of T_H17 cells is determined by the type of antigen-producing bacteria (Yang et al., 2014). These findings further demonstrate that the initiation of T_H17 responses and inflammatory diseases of the intestinal mucosa are closely related to the presence of commensal flora.

Induction of the transcription factors, retinoic acid-related orphan receptor (ROR)-γt and ROR-α, by appropriate antigen presenting cell stimulation with TGF-β and IL-6 or IL-21 drives T_H17 cell differentiation (Ivanov et al., 2006; Mangan et al., 2006). It is also reported that other transcription factors contribute to cytokine production by T_H17 cells (Sato et al., 2011; Zhang and Kaufman, 2008). Human T_H17 cells can also be generated from $CD161^+$ precursors (Kleinschek et al., 2009). The cytokine IL-23 is not strictly necessary for differentiation of T_H17 cells from naïve T cells which lack the IL-23 receptor (IL-23R), but IL-23 is crucial in the maintenance, expansion and terminal commitment of T_H17 lineage via the STAT3 transcription factor (McGeachy et al., 2009; Stritesky et al., 2008). Genome wide association studies showed that *IL23R* and *STAT3* gene polymorphisms have strong associations with T_H17-mediated IBD pathology (Jostins et al., 2012a). The pathogenicity of T_H17 cells in intestinal inflammation has been attributed to the increased production of proinflammatory cytokines such as IL-17A, IL-17F and IFN-γ, and the suppression of T_{reg} differentiation (Peluso et al., 2007).

T_H17 cells are a heterogeneous population with a relatively unstable phenotype. In a given tissue not all T_H17 cells produce proinflammatory cytokines. An IL-17A-producing cell population co-expressing the regulatory T cell marker, transcription factor FOXP3, is present in the intestinal mucosa of CD patients (Hovhannisyan et al., 2011). In an anti-CD3 induced tolerance model, T_H17 cells transdifferentiated and acquired regulatory T cell

functions in the small intestine with increased IL-10 production and down-regulated TNF-α and IL-2 secretion, but not IL-17A (Esplugues et al., 2011). Besides sharing phenotypic and functional similarities with T_{reg} cells, some T_H17 cells also produce the T_H1 cytokine IFN-γ. In an experimental autoimmune encephalomyelitis model, T_H17 cells shut off IL-17 production and become IFN-γ producing cells (Hirota et al., 2011). It has been shown that TGF-β is necessary for sustained production of IL-17F and IL-17A, whereas IL-23 and IL-12 suppress IL-17A but stimulate IFN-γ production in a STAT4 and T-bet dependent manner (Lee et al., 2009a; Lee et al., 2009b). T_H17 cells are also actively cross-regulated by cytokines produced by T_H1 and T_H2 cells, and vice versa. T_H1- and T_H2-associated cytokines like IFN-γ and IL-4 can inhibit T_H17 cell differentiation and cytokine production, while IL-17A suppresses T_H1 cell polarization by inhibiting IFN-γ production (Nakae et al., 2007; Park et al., 2005). Although T_H17 cells have a plastic phenotype, T_H17 proinflammatory cytokines play important roles during intestinal infection and non-infectious inflammation.

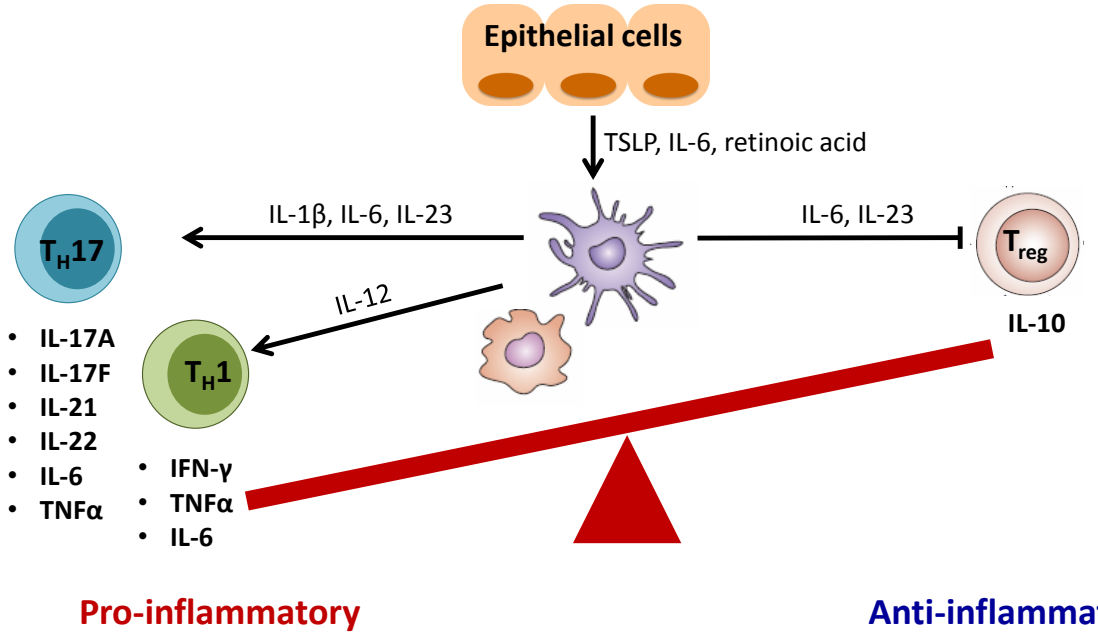


Figure 1.3 Under local inflammation, the balance between pro- and anti-inflammatory cytokines favours an inflammatory response. Under healthy conditions, epithelial cell-derived TSLP, IL-6 and retinoic acid condition APCs towards an anti-inflammatory phenotype. Following interactions with luminal bacteria, APCs secrete cytokines to directly induce an inflammatory T_H1/T_H17 response, while T_{reg} functions are suppressed. IL-23 may play a role in restraining T_{reg} function and thus propogate intestinal inflammation (Brand, 2009).

1.2.2 T_H17-associated cytokines in IBD pathogenesis

T_H17-associated cytokines are highly upregulated in patients with IBD suggesting their pivotal role in intestinal inflammation. For example, *IL17* mRNA expression is increased in the mucosa of IBD patients (Kobayashi et al., 2008; Nielsen et al., 2003; Sugihara et al., 2010) as are serum levels of IL-17A (Fujino et al., 2003; Mohammadi et al., 2013). It is believed that T_H17 cytokines contribute to the amplification and sustention of inflammatory processes by promoting antibody production, chemokine release, neutrophil recruitment and effector T cell activation (Korn et al., 2009). However, the actual functional role of T_H17 cytokines in IBD pathogenesis is not fully understood. A recent clinical trial using secukinumab, an anti-IL-17A monoclonal antibody, demonstrated a lack of efficacy in treating CD patients with active disease (Hueber et al., 2012). Therefore, the role of T_H17 cells in intestinal mucosal inflammation is complex, probably related to their ability to promote epithelial barrier function and fight against extra-cellular pathogens (Troncone et al., 2013). As a result, the physiological function of T_H17-associated cytokines in the intestinal immune system needs further examination.

IL-17A and IL-17F

IL-17A and IL-17F are signature cytokines of T_H17 cells and share the most homologous sequence compared to other IL-17 family cytokines. Their secretion is controlled by RoRγt and STAT3 transcription factors (Ivanov et al., 2006; Yang et al., 2007; Yang et al., 2008b). Both IL-17A and IL-17F can form homodimers or heterodimers and are secreted in combination by activated human $CD4^+$ T cells with all forms functionally active *in vitro* (Wright et al., 2007). Both IL-17A and IL-17F act through the same hetero-dimeric receptor composed of IL-17RA and IL-17RC subunits, with IL-17A exhibiting higher binding affinity compared to IL-17F (Kuestner et al., 2007; Toy et al., 2006). It is clear that IL-17A release can induce myeloid cells to secrete specific chemokines, cytokines, matrix metalloproteinases (MMPs) and antimicrobial peptides which further lead to recruitment and accumulation of neutrophils and local inflammation (Xu and Cao, 2010), whereas the data for IL-17F action are less clear.

IL-17A has been shown to be pathogenic in the context of autoimmune diseases such as multiple sclerosis and rheumatoid arthritis, the effect of IL-17A in the context of chronic intestinal inflammation is more complicated and varies with different mouse models of colitis. Neutralizing IL-17A reduces the experimental colitis induced in a model

of regulatory deficiency with conditional deletion of Stat3 in Foxp3^{+} T_{reg} cells (Chaudhry et al., 2009). Yet in another regulatory deficiency model with IL-10-deficiency, blockade of IL-17A by monoclonal antibody was insufficient unless IL-6 was also neutralized (Yen et al., 2006). Contradictory findings have been reported in different chemically-induced colitis models. IL-17A$^{-/-}$ mice have less severe TNBS colitis compared to wild type mice (Jin et al., 2012), while in contrast another study found that IL-17A neutralization results in more severe colitis in mice treated with dextran sodium sulphate (DSS) (Ogawa et al., 2004; Yang et al., 2008a). Providing further evidence of a non-pathological role for IL-17 in experimental colitis, in a T cell dependent colitis model, lymphopenic host (*Rag1*$^{-/-}$) mice transferred with IL-17A-deficient naïve T cells develop exacerbated colitis compared to recipients receiving wild-type (WT) T cells. Increased IFN-γ levels were found in the mucosa of mice lacking IL-17 signals indicating that IL-17A may exert protective properties by antagonizing production of the pro-inflammatory cytokine, IFN-γ (O'Connor Jr et al., 2009). Thus, while IL-17A can contribute to immunopathology in colitis, this seems to be dependent on the specific nature of the inflammatory response.

Although IL-17F signals through the same receptor as IL-17A, its role in the mucosal immune system is less clear. IL-17F deficiency leads to reduced DSS colitis compared to wild type mice (Yang et al., 2008a). However, IL-17F-deficient T cell transfer induces indistinguishable colitis compare to WT T cell recipients (Leppkes et al., 2009). SCID mice receiving anti-IL-17A and anti-IL-17F monoclonal antibody co-treatment had decreased histological colitis compared to mice receiving isotype control in a transfer colitis model (Wedebye Schmidt et al., 2013), indicating that IL-17F could play a redundant role to IL-17A in mediating intestinal inflammation. More studies are needed to elucidate the unique and overlapping roles of IL-17A and IL-17F and better understand their respective roles in the etiology of IBD.

IL-17C

IL-17C is an IL-17 cytokine family member that is selectively induced by bacterial exposure and inflammatory stimuli (Song et al., 2011). IL-17C binds to a receptor complex consisting of the receptors IL-17RA and IL-17RE, which was preferentially expressed on epithelial cells (Chang et al., 2011). In contrast to IL-17A and IL-17F which are mainly produced by immune cells, IL-17C is distinctly produced by epithelial cells. IL-17C promotes inflammatory responses in the epithelial system by stimulating the expression of

proinflammatory cytokines, chemokines and antimicrobial peptides, which were similar to those induced by IL-17A and IL-17F (Ramirez-Carrozzi et al., 2011). IL-17C and IL-17RE signalling exerted protective functions in dextran sodium sulfate colitis model. Mice deficient in *Il17re* had increased body weight loss and more severe mucosal damage (Ramirez-Carrozzi et al., 2011). In a recent study, IL-17C has been shown to promote colon cancer development by increasing intestinal epithelial cell survival (Song et al., 2014). In summary, IL-17C plays an essential autocrine role in regulating innate epithelial immune responses.

IL-21

IL-21 is a cytokine mostly produced by activated Th17 cells, not APCs (Parrish-Novak et al., 2002). IL-21 signals through IL-21 receptor (IL-21R) consisting of the common receptor γ-chain and IL-21Rα which is widely expressed on immune cells and non-immune cells (Ozaki et al., 2000). IL-21R expression on intestinal epithelial cells is higher in IBD patients compared to healthy controls. An *in vitro* study has shown that IL-21 acts on epithelial cells to induce production of T-cell chemo-attractants (Caruso et al., 2007). This cross-talk between epithelial cells and immune cells demonstrates how IL-21 may perpetuate intestinal inflammation. IL-21 can markedly enhance $CD4^+$ and $CD8^+$ T cell proliferation and counteract Treg differentiation (Peluso et al., 2007; Zeng et al., 2005). Enhanced IL-21 production is found in the mucosa and lamina propria mononuclear cells of IBD patients, likely contributing to the sustained T_H1 immune response in CD patients (Monteleone et al., 2005a). Murine IL-21-deficient naïve T cells failed to differentiate into IL-17A producing cells under T_H17-polarizing conditions *in vitro* and IL-21-deficient mice are protected from DSS colitis showing that IL-21 signalling is necessary to generate the Th17 cells which are involved in DSS-induced intestinal inflammation (Fina et al., 2008). Although murine studies have demonstrated that IL-21 is a critical regulator of T_H17 responses and inflammation in the gut, there are no clinical studies targeting IL-21 in patients with IBD.

IL-22

IL-22 is a member of the IL-10 cytokine family mainly produced by activated effector $CD4^+$ T cells and ILCs. It acts on the IL-22 receptor (IL-22R) comprising IL-10R2 and the IL-22R1 subunit (Wolk et al., 2004; Xie et al., 2000) which is only expressed on non-immune cells, particularly epithelial cells in the skin and mucosal surfaces (Sabat et al.,

2014). IL-22 is a bifunctional cytokine that exerts both tissue protective and pro-inflammatory effects against pathogens in the intestinal mucosal system. IL-22 is involved in tissue repair and in wound healing by enhancing antimicrobial defensin secretion (Wolk et al., 2006). IL-22 plays an important role in the early phase of host defence against *Citrobacter rodentium* infection (Zheng et al., 2008). *Citrobacter rodentium* infection-induced death in genetically obese and high-fat diet fed mice can be rescued by exogenous IL-22 administration (Wang et al., 2014). IL-22-deficient mice had much greater body weight loss and a higher rate of mortality in DSS colitis (Sugimoto et al., 2008). IL-22 sourced from both innate and adaptive immunity also plays a protective role in T transfer-induced colitis (Zenewicz et al., 2008). Furthermore, IL-22 is increased in the mucosa of mice with colitis and up-regulated in patients with Crohn's disease (Brand et al., 2006). IL-22 signalling can increase expression of proinflammatory genes, which may further stimulate intestinal epithelial cell migration and defensin production (Brand et al., 2006). Local IL-22 gene delivery to colonic epithelial cells induces mucus production and goblet cell reconstitution in a murine model which leads to rapid amelioration of local intestinal inflammation (Sugimoto et al., 2008). Another approach to enhance IL-22 production is through the aryl hydrocarbon receptor (AhR) activation. Studies using the AhR agonist, FICZ (6-formylindolo(3,2-b)carbazole), have demonstrated efficacy in preventing and treating multiple forms of experimental colitis through promotion of IL-22 production (Monteleone et al., 2012; Monteleone et al., 2011c). Overall, evidence to date demonstrates that IL-22 is important for maintaining intestinal barrier integrity by improving barrier function and accelerating wound healing.

IL-23

IL-23, a heterodimeric cytokine composed of p40 and p19 subunits, is produced by macrophages, DCs, monocytes and endothelial cells upon antigen activation by ligands for pattern recognition receptors (eg. TLR2 and NOD2), prostaglandin E2 and CD40-ligand (Re and Strominger, 2001; Sheibanie et al., 2004; van Beelen et al., 2007). IL-23 binds to the IL-23 receptor which is composed of IL-12Rβ1 (shared with IL-12 receptor) and IL-23R subunits (Parham et al., 2002). *IL23R* polymorphisms have been associated with IBD susceptibility (Jostins et al., 2012a). IL-23R is widely expressed on innate and adaptive immune cells such as memory T cells, NK cells, DCs, macrophages and innate lymphoid cells (Buonocore et al., 2010b; Geremia and Jewell, 2012; Re and Strominger, 2001). In

response to IL-23 binding, STAT-JAK signalling is activated with predominant activation of STAT3 (Re and Strominger, 2001).

Murine studies have shown that IL-23 is a key cytokine driving intestinal inflammation. In a *Helicobacter hepaticus* induced T cell-dependent colitis, IL-23 not IL-12 has been shown to be the key driver of pathogenesis in intestinal inflammation (Kullberg et al., 2006). Spontaneous intestinal inflammation in IL-10 deficient mice can be ameliorated by specifically neutralizing IL-23 (ie. not also IL-12) with a monoclonal antibody against the p19 subunit (Yen et al., 2006). Transgenic mice over-expressing the p19 subunit of IL-23 develop multi-organ inflammation including intestinal inflammation (Wiekowski et al., 2001). Furthermore, IL-23 deficiency (but not IL-12 deficiency) in *Rag1*$^{-/-}$ recipients is protective in T cell transfer-induced colitis (Hue et al., 2006). IL-23 may suppress Treg differentiation from naïve T cells suggesting intestinal inflammation with high IL-23 production could develop due to reduced immunosuppressive functions (Izcue et al., 2008). IL-23 can activate innate lymphoid cells in *Rag1*-deficient mice to produce IL-17 and induce mucosal inflammation (Uhlig et al., 2006). IL-23 has also been reported to work on hematopoietic stem and progenitor cells by stimulating extramedullary hematopoiesis which results in accumulation of inflammatory monocytes and neutrophils in the inflamed intestine (Griseri et al., 2012). Supporting this evidence, administration of an IL-23p19 vaccine which induces antibodies selectively blocking IL-23 ameliorates TNBS-induced chronic murine colitis (Guan et al., 2013).

Despite the pathogenic role of IL-23 in chronic intestinal inflammation, IL-23 is a critical element of intestinal immunity. For example, IL-23 is necessary for host protection against the attaching and effacing intestinal bacterial pathogen, *Citrobacter rodentium*, in mice (Mangan et al., 2006). Consequently, therapeutic blockade of IL-23 may result in increased susceptibility to enteric pathogen infections. Administration of antibodies against p40 (both IL-12 and IL-23) has proved effective in IBD patients in clinical trials (Mannon et al., 2004; Sandborn et al., 2012; Tuskey and Behm, 2014), while the efficacy of antibodies against p19 (IL-23 only) is still under study.

1.3. Inflammatory Bowel Diseases

IBD, typified by CD and UC, are remitting/relapsing chronic inflammatory disorders of the gastrointestinal tract which appear to result from inappropriate mucosal immune

responses in intestinal tissues to the presence of normal luminal microbes (Podolsky, 2002). CD and UC have many similarities, but there are also several distinguishing pathological differences between them. Both CD and UC have a disease incidence of 10-200 cases per 100,000 persons in western countries with a trend to progressively increasing incidence. The disease prevalence is high especially in North America and Europe, and is now increasing rapidly in Asia, which suggests western lifestyle and environment might be relevant to the disease incidence (Bouma and Strober, 2003). Besides the environmental elements, genetic factors also play an important role in disease prevalence. IBD concordance in monozygotic twins is less than 50% in CD and less than 20% in UC (Halfvarson et al., 2003).

CD can affect any part of the gastrointestinal tract, most commonly, the terminal ileum, cecum and large bowel. A feature of CD is discontinuous lesions with apparently normal intestinal tract separating inflamed regions. Disease histology is characterized by mucosal inflammation, substantial infiltration by T cells and macrophages typically penetrating transmurally, and the presence of granulomas and submucosal fibrosis and structuring. Clinical complications of patients with CD such as diarrhoea, abdominal pain, bowel obstruction and abscess formation are observed (Kaser et al., 2010).

The inflammatory process of UC usually involves the rectum and extends toward the proximal colon in a continuous fashion, but is usually limited to the colon. In contrast with CD, inflammation in UC typically affects only the superficial mucosal layer with loss of goblet cells, lymphocyte infiltration, surface ulceration and crypt abscesses. Patients with UC often experience severe diarrhea and intestinal bleeding. Defects in peristaltic function and the development of a rigid colonic tube can present in patients with severe UC. Chronic uncontrolled inflammation in UC increases the risk of colon carcinoma by approximately 10-fold (Kaser et al., 2010).

1.3.1 Genetic factors involved in IBD

Genome-wide association studies (GWAS) have revealed more than 163 genes are linked to the susceptibility of IBD, including amongst the genes with the highest relative risk, genes related to T_H17 immunity (*IL23R*, *IL12B*, *STAT3, JAK2 and CCR6*), IL-10 signaling (*IL27*, *IL10* and *IL10Ra*), epithelial function (*ERRFI1* and *HNF4α*) and innate sensors (*NOD2*, *CARD9 and ATG16L1*) (Jostins et al., 2012a; Khor et al., 2011). As this

project focuses on the T cell immune response, particularly the T_H17 response, gene variants associated with innate and adaptive T_H17 arm immunity will be discussed.

NOD2 is the strongest susceptibility locus for CD and it is particularly important in intestinal innate immunity and cells of the macrophage/monocyte lineage, dendritic cells and epithelial cells. Nucleotide-binding oligomerization domain containing 2 (NOD2) is a cytoplasmic protein product of *NOD2* that acts as a pathogen recognition receptor sensing virus and all bacteria except mycobacteria and chlamydia (Girardin et al., 2003; Inohara et al., 2003). Mutation of *NOD2* in patients with CD may lead to impaired autophagy induction which leads to dysregulated bacteria translocation and failed antigen presentation in the lamina propria dendritic cells (Cooney et al., 2010). Functional mutations in both alleles of *NOD2* gene increases the susceptibility of CD by more than 30-fold (Cuthbert et al., 2002), making *NOD2* the most powerful mutation found in CD.

Genome-wide association studies demonstrate that an uncommon coding variant of the *IL23R* gene (rs11209026, c. 1142G> A, p. Arg381Gln) conferred strong association with both CD and UC (Duerr et al., 2006a; Jostins et al., 2012b; Zindl et al., 2013). Interestingly, some *IL-23R* SNPs are associated with increased disease risk, while others indicate decreased disease susceptibility (Ebrahimi Daryani et al., 2014; Lacher et al., 2010; Lauriola et al., 2011; Mihaljevic et al., 2013; Pidasheva et al., 2011; Sarin et al., 2011; Sarlos et al., 2014). Although the precise mechanism of the *IL23R* polymorphisms regulating IBD susceptibility is not fully understood, the current understanding is that the protective *IL23R* polymorphism is associated with impaired IL-23R signalling and reduced STAT3 phosphorylation in T cells, which contributes to reduced proinflammatory cytokine production (Cho, 2008; Pidasheva et al., 2011).

The *IL12B* gene which encodes the p40 shared-subunit of heterodimeric cytokines IL-12 and IL-23 (Oppmann et al., 2000), has been linked with CD (Barrett et al., 2008a; Ferguson et al., 2010a), which further suggest that IL-12/IL-23 pathway plays a pathogenic role in chronic intestinal inflammation.

The *STAT3* gene (encodes signal transducer and activator of transcription 3) and *JAK2* gene (encodes Janus kinase 2) encode signalling proteins downstream of IL-23R, but also activated by other cytokines such as IL-6, IL-10, IL-22 and IL-26 (Brand, 2009). It

has been shown that the genetic variants of the *JAK2* and *STAT3* genes have a strong association with IBD susceptibility (Barrett et al., 2008b; Ferguson et al., 2010b; Polgar et al., 2012). STAT3 has a central role in T_H17 cell differentiation, and its activation by IL-23 induces downstream proinflammatory IFN-γ and IL-17 production (Watford et al., 2004), which further suggest that the STAT3-JAK2 signalling pathway is integral to IBD pathogenesis.

Evidence from GWAS strongly suggests that the IL-23 signalling pathway and T_H17-associated cytokines play an important role in IBD pathogenesis. Therefore, targeting cytokines or key components of this signaling pathway provide promise for treatment of IBD.

1.3.2 Cytokines involved in the pathogenesis of IBD

As an immune disorder characterized by a dysregulated inflammatory T cell response, elevated pro-inflammatory cytokine levels are often observed in patients with IBD. At the same time, production of regulatory cytokines such as TGF-β and IL-10 is decreased, indicating the balance between pro and anti-inflammatory responses is broken.

Mucosal cytokine profiles determine the local T cell differentiation environment and may play an important role in the IBD pathogenesis. Cytokines from different T helper cell subsets are elevated in the inflamed mucosa of patients with UC or CD (Christophi et al., 2012; Kobayashi et al., 2008; Leon et al., 2009). Some T_H1 related cytokines including IFN-γ, IL-12 and TNF-α are overexpressed in the inflamed mucosa of both UC and CD patients (Christophi et al., 2012; Leon et al., 2009; Olsen et al., 2007). With the discovery of T_H17 cells, now it is well accepted that IBD is associated with increased inflammatory cytokine production by multiple effector T cells, including T_H17 cells. T_H17-associated cytokines such as IL-17A, IL-17F, IL-21 and IL-22 are excessively produced in both CD and UC (Monteleone et al., 2011a; Monteleone et al., 2011b).

IL-17A is the signature cytokine produced by T_H17 cells, and demonstrates strong proinflammatory functions (see 1.2.2 for details). The average number of IL-17-producing cells is significantly increased in patients with active UC and CD, and mRNA gene expression and serum levels of IL-17A are also elevated in patients with IBD (Fujino et al., 2003). Multiple cell types including δγT cells and innate lymphoid cells are the source of

IL-17A in the mucosa of IBD patients (Martin et al., 2009; Spits and Di Santo, 2011). mRNA expression of another T_H17 effector cytokine, IL-17F, is also augmented in the inflamed lesions of CD (Seiderer et al., 2008), indicating that IBD is associated with an altered immune balance favoring an inflammatory T_H17 response.

IL-21 expression is higher in the mucosa of patients with CD and UC compared to healthy controls. In IBD most IL-21 is produced by IFN-γ producing $CD4^+$ T cells rather than IL-17A producing cells. Neutralizing IL-21 in LPMC isolated from CD patients induces decreased expression of the T_H1 associated transcription factors, STAT4 and T-bet (Caruso et al., 2007; MacDonald and Monteleone, 2005). Murine studies have demonstrated that IL-21 regulates IL-17 production by T_H17 cells in chemically-induced murine colitis (Fina et al., 2008). Therefore IL-21 may contribute to IBD pathogenesis through augmentation of both inflammatory T_H1 and T_H17 responses.

Increased IL-22 gene expression in mucosal lesions and elevated serum cytokine levels are observed in both CD and UC patients (Brand et al., 2006; Schmechel et al., 2008), which together with the roles for IL-22 shown in mouse models (see 1.2.2 for details), suggests that IL-22 may contribute to epithelial healing, defensin production and suppression of local inflammation.

IL-23 gene expression is upregulated in both UC and CD. Its upregulation correlates with increased IL-17A production in UC and IFN-γ secretion in CD, although T_H17 cells exist in both diseases (Kobayashi et al., 2008). As IL-23 is the key cytokine driving T_H17 cell differentiation and cytokine production, its elevation is consistent with the documented increase of proinflammatory cytokines such as IL-17 and IFN-γ in patients with IBD.

1.3.3 Current treatments for IBD

Corticosteroids

Conventional corticosteroids such as prednisolone have been used to treat IBD for over 50 years and are the preferred initial treatment for patients with IBD. Corticosteroids are able to down-regulate proinflammatory cytokine production by effector T cells, reduce inflammation levels and effectively induce remission in patients with CD (Cottone et al., 2011). Corticosteroids rapidly and effectively lead to short term clinical remission, but long

term use is limited by high relapse rates and side effects related to long term glucocorticoid use (Faubion et al., 2001). Corticosteroid treatment to induce remission may be less effective than combined immunosuppressive drugs in patients with newly diagnosed CD (D'Haens et al., 2008). Long-term use of corticosteroids is also associated with numerous systemic adverse events including opportunistic infections, diabetes mellitus, hypertension and increased fracture risk (Curkovic et al., 2013).

Thiopurines

The thiopurine drugs 6-Mercaptopurine (6-MP) and its pro-drug azathioprine (AZA) are purine analogues that competitively interfere with nucleic acid metabolism. Both drugs have immune modifier properties as a result of reducing leukocyte proliferation (Lennard, 1992). Although many controlled clinical trials have investigated the efficacy of thiopurines to treat moderate-to-severe IBD patients, results have been conflicting and controversial. Meta-analysis of eight randomized placebo controlled trials of 6-MP and AZA therapy on patients with CD revealed thiopurine treatment is more effective than placebo for induction of remission in patients with active CD (Prefontaine et al., 2010). Combination therapy with thiopurines and anti-TNF antibodies improves response rates compared to anti-TNF antibody alone (Colombel et al., 2010; Panaccione et al., 2011). However, another meta-analysis of five parallel-group randomized controlled trials failed to demonstrate that AZA/6-MP treatment alone has statistically significant benefit to induce remission in active CD and UC (Khan et al., 2011).

Some severe side effects have been associated with thiopurine treatment, alone or in conjunction with corticosteroids, resulting in cessation of therapy in up to 25% of patients (Lakatos and Kiss, 2011). General or mild side effects include nausea, abdominal pain and flu-like symptoms, while severe side effects include suppression of white blood cell and platelet production, pancreatitis and hepatitis (Prefontaine et al., 2010). The strong immunosuppressive functions of thiopurines may also result in severe systemic infections and malignancy such as hepatosplenic T cell lymphomain some patients (Issa et al., 2007; Karran and Attard, 2008; Schneeweiss et al., 2009).

5-aminosalicylic acid

5-aminosalicylic acid (5-ASA) has been used for the treatment of IBD especially UC for many years. Although 5-ASA can be used in combination with steroids to induce and maintain remission in patients with IBD (Pithadia and Jain, 2011), the exact mechanism of actions are still unclear. Several potential mechanisms have been suggested, including prevention of mucosal leucocyte proliferation and trafficking, inhibition of IL-1β and TNF-α production, inhibition of the lipoxygenase pathway, the scavenging of free radicals and oxidants, and inhibition of NF-κB transcription factor pathway (van Bodegraven and Mulder, 2006). While 5-ASA has long-established efficacy in treating UC (Hanauer et al., 1993), the efficacy of 5-ASA in treating CD is less promising. A recent meta-analysis concluded that the role of 5-ASA in inducing remission in active CD and maintaining remission in CD remains uncertain (Ford et al., 2011). Side effects of 5-ASA, including headache, nausea and fatigue, happen in about 10 to 45% of patients. These reactions can be minimized by decreasing the dose. However, adverse effects occur in some patients, including severe allergic reactions, fever, hepatitis, pneumonitis, hemolytic anemia, and bone marrow suppression (Pithadia and Jain, 2011).

Biological therapy

Lack of significant efficacy of conventional treatments in IBD and better understanding of disease pathology has led to new therapies designed to target specific molecules of inflammatory response - the so called biological drugs.

Patients that fail to respond to conventional steroids and immunosuppressive drugs are usually treated with anti-tumour necrosis factor-α (TNF-α) antibodies. Infliximab is a chimeric monoclonal antibody with proven efficacy in improving clinical response and maintaining remission in patients with refractory luminal CD. Infliximab rapidly and profoundly induces endoscopic healing (Hanauer et al., 2002; Present et al., 1999; Targan et al., 1997). There are multiple proposed mechanisms of actions of anti-TNF antibodies including neutralization of soluble TNF-α, induction of lamina propria T cell apoptosis, $CD68^+$ macrophage apoptosis and decreasing IL-17 expression (Caprioli et al., 2013; ten Hove et al., 2002). In a randomized, open-label study in paediatric patients with moderately-to-severe active CD, 10 weeks of infliximab demonstrated a clinical response rate of 88.4% ($p<0.05$ vs placebo) and remission rate of 58.9% ($p<0.05$), and a response rate of 63.5% ($p=0.002$) and remission of 55.8% ($p<0.001$) after one year of treatment

(Hyams et al., 2007). Infliximab also has demonstrated efficacy in patients with UC. Patients receiving 5mg and 10mg of infliximab both had increased clinical responses (69% and 61%, respectively) compared to those receiving placebo (37%, P<0.001 for both comparisons) (Rutgeerts et al., 2005). Other anti-TNF monoclonal antibodies – adalimumab, certolizumab pegol and golimumab have also shown clinical benefits for active IBD patients (Colombel et al., 2007; Hanauer et al., 2002; Peyrin-Biroulet et al., 2007; Sandborn et al., 2014; Trinder and Lawrance, 2009).

Despite the efficacy of TNF antagonists in IBD there are considerable issues with respect to safety and loss of response. The safety issues of anti-TNF treatment include risk of infection (resulting in tuberculosis, sepsis and opportunistic infection), malignancy, autoimmunity and paradoxical inflammation (Billiet et al., 2014). Among these side effects, increased risk for opportunistic infections is the most prominent issue. Several safety analyses on pooled-trial data found increased infections in anti-TNF-treated patients compared to the placebo control group (Burmester et al., 2013; Fidder et al., 2009; Lichtenstein et al., 2012a; Lichtenstein et al., 2012b). Besides the side effects, non-response limits the use of anti-TNF antibody in clinical treatment. Approximately one third of patients do not respond to anti-TNF therapy (primary non-response) (Hanauer et al., 2006; Sandborn et al., 2007a; Targan et al., 1997). Within the initial responders, approximately 40% of patients will develop non-response or intolerance during treatment (secondary nonresponse), and require either dose escalation or switching to another anti-TNF antibody (Colombel et al., 2007; Hanauer et al., 2002; Schreiber et al., 2007). Secondary non-response patients treated with class-switched anti-TNF antibodies generally have lower response rates compared to patients naïve to anti-TNF antibody treatment (Billiet et al., 2014; Sandborn et al., 2007b).

IL-6 has been shown to play a pivotal role in the development of CD, both in animal colitis models and human studies (Atreya and Neurath, 2008; Yamamoto et al., 2000). Several monoclonal antibodies against IL-6 or IL-6 receptor (IL-6R) have been developed and tested in inflammatory diseases. Tocilizumab, a humanized anti-IL-6R monoclonal antibody, was developed initially to treat patients with autoimmune disease such as rheumatoid arthritis (Ding and Jones, 2006), but also has shown efficacy in treating patients with active CD. In a placebo-controlled phase II study, 80% of patients treated with Tocilizumab every two weeks had a clinical response compared to 31% of the

placebo-treated patients (P=0.019), and 20% of treated patients went into remission compared to 0% of the control group (Ito et al., 2004). Another fully humanized anti-IL-6 monoclonal antibody PF-04236921 is being tested in two ongoing trials in CD patients who failed anti-TNF therapy. However, a recent trial using a novel humanized anti-IL-6 monoclonal antibody BMS-945429 to treat active CD patients was prematurely discontinued due to severe intestinal perforations. Based on these results, targeting IL-6 in IBD needs further careful investigation.

Three clinical trials have evaluated the efficacy of fontolizumab, a humanized anti-IFN-γ monoclonal antibody, in patients with moderate-to-severe CD. Although fontolizumab was well tolerated, a significant clinical response was not observed compared to the placebo group (Hommes et al., 2006; Reinisch et al., 2010; Reinisch et al., 2006). Therefore even though CD has a prominent T_H1 response, neutralization of the main T_H1 cytokine does not reverse pathology.

Two fully humanised IgG1 monoclonal antibodies against the p40 subunit of IL-12/23 cytokines have been developed: briakinumab and ustekinumab. A randomized study of briakinumab on 79 moderate-to-severe CD patients demonstrated that weekly subcutaneous injections of 3mg/kg body weight of anti-p40 antibody induced a higher clinical response rate compared to the placebo group at week 7, but not week 18 (Mannon et al., 2004). However, the remission rate was not significantly different between anti-p40 antibody and placebo treated groups (Mannon et al., 2004). Later a double-blinded, placebo controlled, parallel-group cross-over trail of ustekinumab also exhibited some efficacy in treating patients with moderate-to-severe Crohn's disease, with an increased clinical response rate at week 4 and week 6 after cross-over of treatments, but failed to reach the primary end-point at week 8 (Sandborn et al., 2008). A recent phase 3 clinical trial of ustekinumab has been carried out on patients suffering from moderate-to-severe CD who had previously failed anti-TNF therapy (Sandborn et al., 2012). Ustekinumab demonstrated significant benefit in inducing clinical responses compared to placebo, but not remission. In a subset of patients with an initial response to ustekinumab, further treatment with ustekinumab as a maintenance therapy again significantly increased clinical response rates compared to placebo (Sandborn et al., 2012). Taken together, these results show that anti-p40 antibody treatment is effective as an induction and maintenance therapy in subsets of CD patients. This might be particularly useful for patients who do not

respond to anti-TNF therapy or are intolerant to anti-TNF drugs. To date, these antibodies haven't been tested or trialed in UC. Other large clinical trials with ustekinumab are underway which should help to define anti-p40 antibody efficacy in different treatment settings.

In a double-blind, placebo-controlled clinical trial, patients with moderate-to-severe CD was treated with secukinumab, a humanized anti-IL-17A monoclonal antibody. However, secukinumab failed to demonstrate efficacy and blockade of IL-17A exacerbated CD in a subset of patients compared to placebo (Hueber et al., 2012). Another anti-IL-17A antibody, AMG827, was also ineffective in treating active CD patients. Evidence of disease worsening was observed compared to the placebo control group (Targan et al., 2012), indicating that targeting this single cytokine of the T_H17 pathway is not sufficient to suppress intestinal inflammation. It is possible, based on mouse data (O'Connor Jr et al., 2009), that neutralizing IL-17A fosters T_H1 cells differentiation and promotes production of IFN-γ. In contrast to the antibody therapy targeting only IL-17A, Vidofludimus (SC12267), a small molecule targeting the JAK/STAT and NF-κB pathway and suppressing the expression of proinflammatory cytokines IL17A, IL17F and IFN-γ is efficacious in treating IBD (Herrlinger et al., 2013).

Besides the current antibody treatment against specific inflammatory cytokines, other biologics as treatment have also been tested in clinical trials such as recombinant human IL-10, granulocyte-macrophage colony stimulating factor (GM-CSF), antibodies against leukocyte migration and drugs which induce T cell apoptosis. Vedolizumab, an antibody against α4β7 integrin which blocks lymphocyte trafficking, has shown efficacy as induction and maintenance therapy for UC (Feagan et al., 2013). However, vedolizumab did not show substantial efficacy in treating active CD patients (Sandborn et al., 2013).

In conclusion, the most common treatment for IBD clinically is "step-up" which includes corticosteroids to induce remission and thiopurines or anti-TNF antibody treatment to maintain remission. Additional therapies targeting proinflammatory cytokines or combinations of different biologics to block the inflammatory cascade are needed for moderate-to-severe patients with IBD. Preferably these biologics should do this without substantially increasing the risk of infection.

Surgery

Surgery plays a limited/last resort role in the management of IBD. It is indicated for patients with medical therapy failure, intolerable side effects of medications, toxic megacolon, perforation, high-grade or multifocal dysplasia and uncontrollable bleeding (Cohen et al., 2005). Total proctocolectomy may be curative for patients with isolated UC, while a high rate of surgical complications is noted in patients with CD. Therefore, surgery usually is restricted to patients with IBD that are refractory to other treatment options.

1.4. Murine experimental models of colitis

1.4.1 Colitis occurring as a consequence of gene mutations or introduction of transgenes

In mice a wide range of genetic defects (gene knock-out or mutations) develop spontaneous intestinal inflammation. These models include epithelial restricted knockouts (Muc2, Xbp1, Agr2, NEMO and fatty acid synthase deficient mice) (Heazlewood et al., 2008; Kaser et al., 2008; Nenci et al., 2007; Park et al., 2009; Van der Sluis et al., 2006; Wei et al., 2012), regulatory cell defects (IL-10 deficient mice, IL-2 deficient mice and TGF-β deficient mice) (Kuhn et al., 1993; Sadlack et al., 1993; Shull et al., 1992), defects causing increased effector cell function (*Tnf* mutant mice and *Stat4* transgenic mice) (Kontoyiannis et al., 1999; Wirtz et al., 1999) and defects in antigen presentation (MHC II-deficient mice) (Grusby and Glimcher, 1995). These models are useful when studying the effects of gene deficiency in the development of colitis and inflammation. However, some of the gene knockout mice have widespread immunopathology limiting their relevance to the type of inflammation present in IBD.

1.4.2 Colitis induced by exogenous chemicals

Exogenous chemical toxins such as DSS and trinitrobenzene sulfonic (TNBS) acid are widely used to induce colitis in mice. Histopathology of mice with DSS colitis usually includes oedema, epithelial damage, ulceration, crypt abscesses, neutrophilic infiltration and submucosal inflammation and this is considered a model for UC (Dieleman et al., 1998). On the other hand the response to the hapten TNBS causes a more T cell mediated colitis that is considered to be CD-like. Chemically-induced colitis models are commonly employed as they are practically easy and achievable. However, this acute inflammation does not truly reflect the real progressive chronic disease process in human IBD, and these models do not provide strong predictive value for testing drugs applicable to human disease.

1.4.3 Colitis induced by transfer of naïve T cells into lymphopenic hosts

Chronic colitis induced by transfer of T cells into lymphopenic recipients has been extensively studied and employed as a research model in the past decade (Powrie et al., 1993). It is the best-characterised model of colitis induced by disruption of T cell homeostasis. In this model, $CD4^{+}CD45RB^{high}$ donor T cells are transferred into a lymphopenic host such as SCID or *Rag*-deficient mice. In the absence of regulatory T cells and anti-inflammatory cytokines, donor naïve T cells induce severe colonic and small intestinal inflammation with the time post-transfer to develop colitis dependent on the microbial flora in the animal facility. Histopathological changes in the distal colon include mucosal inflammation, epithelial cell hyperplasia, goblet cell depletion, polymorphonuclear and mononuclear cell infiltration, crypt abscesses and epithelial erosion. Reconstituted mice exhibit varying degrees of diarrhoea, rectal bleeding, and body weight loss depending on the strains of donor and recipient mice (Powrie et al., 1993). Advantages of this model are that early T cell-mediated immune responses and inflammation can be examined, and it can be employed to study the effects of general immunological treatments on colitis. It is also a useful model to investigate the suppressive functions of regulatory T cells in limiting the onset and progression of inflammation (Ostanin et al., 2009). However, colitis induced by adoptive T cell transfer develops in a severely lymphopenic host with an incompetent immune system, and IBD patients are not lymphopenic. Therefore, although a useful tool this scenario does not reflect disease development under physiological conditions with immune surveillance from regulatory T cells.

1.4.4 Winnie mice - a spontaneous model of colitis

Winnie mice, which are used extensively as a model of colitis in this thesis, were generated by ENU mutagenesis and were the first model that has a single missense mutation in the *Muc2* mucin gene. This mutation results in a proportion of the Muc2 protein misfolding and accumulating in the endoplasmic reticulum (ER) which results in ER stress in intestinal goblet cells, a depleted intestinal mucus barrier and spontaneous colonic inflammation (Heazlewood et al., 2008). The important feature of this model is that the *Winnie* mice have an epithelial defect with normal underlying immunity, making this substantially different to most models of IBD.

Our laboratory has fully characterized the nature of the inflammatory response in *Winnie* mice (Eri et al., 2011). Immunophenotyping and analysis of expression/levels of cytokines from mesenteric lymph nodes (mLNs) were assessed in *C57BL/6* and *Winnie* mice. Immunophenotyping of colonic LPMCs showed an increased proportion of $CD11c^+$ APCs and a 4-fold increase in activated DCs ($CD11c^+$ MHC class II^{hi}) in *Winnie* accompanied by decreased production of the APC conditioning factor TSLP. Upon LPS stimulation, APCs from *Winnie* mice secreted elevated levels of IL-12/23p40, IL-6 and MIP-1α compared to the *C57BL/6* mice suggesting a T_H17 polarization. *Winnie* mice also displayed a significant increase in the expression of T_H17 signature genes *Il17a, Il17f, Tgfb* and *Ccr6* in the intestinal mucosa and marked increases in IL-1β, particularly in the distal colon compare to *C57BL/6*. Intestinal mLN leukocytes isolated from *Winnie* mice secreted multiple T_H1, T_H2 and T_H17 cytokines upon stimulation, with a striking 100-fold augmentation in IL-17A in an age-dependent manner.

Based on *Winnie* mice, our lab further generated *RaW* mice by crossing *Rag1*$^{-/-}$ *with Winnie*, which is a useful model to examine the effect of both effector and regulatory lymphocytes and cytokines in the *Winnie* model of colitis. The absence of T and B lymphocytes in *RaW* mice altered the pattern of inflammation but colitis persisted, particularly in the proximal colon, suggesting that innate immune cells play a role in mediating spontaneous colitis. There was no difference in colitis severity and clinical symptoms between young *Winnie* and *RaW* mice (8 weeks). However, *RaW* mice did not show the progressive increase in clinical symptoms and colitis characteristic of *Winnie* mice as they age. These data indicate that lymphocytes play an active role in progression of colitis in this model (Eri et al., 2011).

In conclusion, the primary epithelial defect and mucosal barrier dysfunction in the *Winnie* mice results in complex multi-cytokine mediated colitis involving both innate and adaptive immune components with a dominant IL-23/T_H17 response which share similarities to patients with IBD. Therefore, the underlying basis for my thesis was that targeting proinflammatory cytokines, especially T_H17-associated cytokines, in *Winnie* mice will deepen understanding of the complex cytokine milieu in intestinal inflammation and hopefully indicate the best therapeutic approaches to manage IBD.

2.0 Rational and Aims

IBD is a chronic relapsing inflammatory disorder without definitive cure. Current medical management of IBD does not treat the cause of disease, and these treatments are only effective in reducing the degree of inflammation and accompanying symptoms, but typically fail to suppress disease progression in the long term. Anti-inflammatory drugs with immune-suppressive functions have shown efficacy in treating IBD which further demonstrates the pathogenic role of lymphocytes and proinflammatory cytokines in disease progression. Although a range of different anti-cytokine antibodies and biologics have been studied in pre-clinical animal models and have in some cases entered clinical trial, only several of them have proved be effective in inducing remission in IBD patients. While these biologic drugs are in widespread use and are efficacious in many patients, some IBD patients remain unresponsive and a substantial proportion loses responsiveness during treatment. Genetic and experimental evidence suggests that the IL-23/T_H17 pathway is involved in IBD pathogenesis. There is enormous interest from researchers and the pharmaceutical industry in targeting the T_H17 immune response to treat IBD. However, trials of IL-17 antagonism have been disappointing and the detailed mechanism of T_H17 inflammation in the intestine is only partially understood, particularly as it involves multiple interacting cytokines and complex cross-talk between epithelial cells and leukocytes.

This project using a unique mouse model of intestinal inflammation, *Winnie* mice, provides an opportunity to investigate the roles of T_H17-associated inflammatory cytokines, especially IL-23 and IL-17A, in the mucosal inflammation setting. The primary epithelial defect and mucosal barrier dysfunction in the *Winnie* mice results in complex multi-cytokine mediated colitis involving both innate and adaptive immune components with a dominant IL-23/T_H17 response which share similarities to patients with IBD. The important feature of this model is that the *Winnie* mice have an epithelial defect with normal underlying immunity, making this substantially different to most models of IBD. Therefore, targeting T_H17 associated cytokines in *Winnie* mice will provide insights into their role within the cytokine milieu in intestinal inflammation and hopefully will suggest improved therapeutic approaches to manage IBD.

2.1. Central Hypothesis

Proinflammatory cytokines, especially IL-23 and T_H17-associated cytokines contribute to the pathogenesis of chronic intestinal inflammation.

2.2. Aims

The hypothesis will be addressed via the following specific aims:

1. To investigate if IL-17A or IL-17Ra signaling in T cells contributes to the intestinal inflammation in the T cell transfer experimental colitis model.

2. To utilize IL-17A cytokine deficient mice to examine the importance of IL-17A for initiation and progression of colitis.

3. To assess the efficacy of monoclonal antibodies against T_H17 effector cytokines or their receptor in suppressing intestinal inflammation.

4. To assess the efficacy of monoclonal antibodies against key T_H17 regulatory cytokine, IL-23, in suppressing intestinal inflammation.

3.0 Materials and Methods

3.1. Cell culture

Three different human colorectal carcinoma cell lines were used, LS174T, LS513 and HT29 (American Type Culture Collection, Manassas, USA). LS174T and HT29 cells were cultured in DMEM with 4.5g/L glucose and 0.11g/L pyruvate; and LS513 cells were cultured in RPMI1640 (Gibco, Life Technologies, NY, USA). Both media were supplemented with 10% FCS, 2 mM L-glutamine and 100U/ml penicillin-streptomycin (Gibco, Life Technologies). Cells were maintained in a 37°C incubator with a humidified atmosphere of 5% CO_2.

3.2. Treatment of cultured cells

Cells were seeded at selected concentrations as specified in individual experiments in 24 or 96 well plates on the day before treatment. After 24 h incubation, cells were treated with various cytokines, recombinant human IL-17A, IL-22 and IL-23 (R&D Systems, MN, US) or various chemicals: tunicamycin (Tm, Sigma-Aldrich, MO, US), ROS inhibitors-reduced glutathione (GSH, Sigma-Aldrich), NFκB inhibitor (BAY11-7085, Enzo Life Sciences, PA, US), Stat3 inhibitor (VI S3I-201), Stat5 inhibitor (573108). All Stat inhibitors were obtained from EMD Millipore Bioscience (CA, US). including The concentrations and specific treatment regimens used for each drug are described later in the results chapters and are indicated in the figure legends. Cytokines were dissolved in PBS and chemicals were dissolved in dimethyl sulphoxide (DMSO), with appropriate vehicle controls used in all experiments.

3.3. Immunofluorescent staining of cells

Cells were seeded on top of poly-D-lysine (0.1mg/ml) pre-coated coverslips in 24-well plates at 1 x 10^6 cells/ml density. Various treatment regimens were carried out 24 h after plating as specified in the figure legends of the results chapters. For immunofluorescence staining, cells were fixed in 4% paraformaldehyde for 15 min at room temperature, followed by permeabilization with ice-cold methanol for 10 min at −20°C. Non-specific binding was blocked with 5% FCS in PBS for 60 min at room temperature. Primary antibodies diluted to specific concentrations in PBS supplemented with 1% BSA were incubated with cells overnight at 4°C. Cells were washed 3 times with PBS before incubation with fluorochrome-conjugated secondary antibodies for 1-2 h at room temperature. Coverslips with cells were mounted to slides with Prolong® Gold Antifade

Reagent with DAPI (Life Technologies). Fluorescent staining was examined using an Olympus BX63® motorized microscope with the cellSens software v.1.7 (Olympus, Tokyo, Japan).

3.4. Quantification of intra-cellular reactive oxygen species concentrations by 2',7'-dichlorodihydrofluorescein diacetate (DCFDA)

LS174T cells were seeded in black-walled 96-well plates with serum free media for at least 12 h before treatment. Cells were then treated as specified in the figure legends for 30 min, and intra-cellular ROS levels were measured. Cells were pulse-labelled with 10 µM DCFDA for 10 min at 37°C, followed by washing with warm serum free DMEM medium. Cytokines and stimuli were added to induce ROS within cells, which oxidize DCFDA into a fluorescent product. After 30 min incubation, fluorescence intensity of oxidized DCFDA was measured using a POLARstar Omega plate reader (BMG Labtech, Ortenburg, Germany) with excitation wavelength at 495 nm and emission wavelength at 525 nm.

3.5. Quantification of oxidative stress by dihydroethidium (DHE) oxidization

To assess the intracellular ROS and oxidative stress level, freshly isolated mouse neutrophils were plated in 96-well plate and immediately treated with 2.5 µM dihydroethidium (DHE, Sigma-Aldrich) solution for up to 2 h at 37 °C. Cells were then washed with room temperature PBS 3 times for 5 min each. In presence of O_2^-, cell-permeable O_2^- scavenger DHE is oxidized to fluorescent ethidium bromide which is then trapped in the nucleus by interaction with DNA. The number of fluorescent nuclei and the intensity of the ethidium fluorescence indicate the relative level of O_2^- production. Fluorescence intensity was measured with excitation at 535 nm wavelength and emission at 610 nm wavelength using a POLARstar Omega plate reader (BMG Labtech).

3.6. Unfolded protein response and ER stress reporter assay

LS174T cells were transfected with pCAX-F-XBP1ΔDBD-*venus* plasmid (splicing of XBP1 messenger RNA [mRNA] by IRE1α results in the translation of Venus-GFP but not an active form of XBP1, a gift from Prof. Masayuki Miura) (Iwawaki et al., 2004) using the Lipofectamine 2000 reagent according to the manufacturer's instructions (Life Technologies). Briefly, cells were seeded 24 h before transfection. Transfection was performed when cells reached 70-90% confluence. One µL of Lipofectamine® 2000

Reagent was diluted in 100 µL of Opti-MEM® Medium and 1 µg of plasmid DNA was diluted in 100 µL of Opti-MEM® Medium. Then the diluted DNA was added to the diluted Lipofectamine® 2000 Reagent (1:1 ratio) and incubated for 15 min at room temperature. The DNA-lipid complex was then incubated with cells for 24 h before replacing the media with complete DMEM medium. Various treatments were carried out 48 h after transfection. Cytokine and drug concentrations are indicated in the figure legends in respective results chapters. Venus-GFP signal was detected using a POLARstar Omega plate reader (BMG Labtech) with excitation wavelength at 485 nm and emission wavelength at 525 nm.

3.7. Flow cytometry

Isolated or cultured cells were resuspended at 1 x 10^6 cells/ml concentration in FACS buffer (PBS with 3% FCS, filter sterilized) before staining. Pre-titrated antibody cocktails were added into 100 µL FACS buffer containing 1 x 10^5 cells and incubated on ice for 30 min in the dark. Cells were then washed 3 times with FACS buffer to remove unbound antibodies. Unstained, isotype antibody stained cells and single colour stained cells were included in each run in order to determining the compensation setting on the machine before acquisition. Cells were analyzed using CyAn™ ADP Analyzer (Beckman Coulter, NSW, Australia) and FlowJo v8.8.6 software.

3.8. Mouse strains and general husbandry

All mouse strains used in the project are listed below in Table 3.1. All animals were housed in PC2 animal facilities including the Mater Annex at the Mater Medical Research Institute and the UQ Biological Resource Facility at the Translational Research Institute. These facilities were free of Norovirus and *Helicobacter pylori* infection. All animal research and experiment protocols were approved by the Animal Ethics Committee of the University of Queensland, and conducted according to National Health and Medical Research Council guidelines (Approval number for the project 205/12, Appendix A).

3.9. Mouse monitoring for experiments

All experimental mice were fed with autoclaved food and water. Sample sizes for each animal experiment were determined by Power analysis based on $\alpha = 0.05$, $\beta = 0.80$ and a change of 2 standard deviations of the mean of the primary outcome measure, colon weight. Mixed genders of mice were used in all experiments. Mice were matched for age and sex between different strains, and mice in the same cage were randomly allocated to

different treatments. For all experiments, the general condition, behavior and bodyweight of mice were closely monitored using the scoring sheet for Intestinal Pathology Assessment in Mice (Appendix B).

Table 3.1 Mouse strains used in the project

Strain	Genotype	Characters	Source
C57BL/6	C57BL/6JArc	Wild type mice	Purchased from Australian Research Council (ARC, Western Australia)
Rag1$^{-/-}$	B6.SVJ129-*Rag1*^{tm1Bal}/Arc	Genetically modified on the *C57/BL6* background, lacking the *Rag1* gene	Purchased from ARC
Winnie	C57BL/6JSfdAnu-Muc2^{wnn}/AnuApb	Genetically modified through N-ethyl-N-nitrosourea mutagenesis on *C57/BL6*, have a single missense mutation in the D3 domain of the MUC2 mucin gene	In house
RaW		*Muc2*$^{wnn/wnn}$*Rag1*$^{-/-}$	In house
IL-17A$^{-/-}$	C57BL/6-Tg(Scgb1a1-Il17a)3Cdon/J	Genetically modified on *C57BL/6* background, *Il17a* gene expression silenced	Amgen Inc. (CA,US)
IL-17A$^{-/-}$ *Winnie*		*Muc2*$^{wnn/wnn}$*IL-17A*$^{-/-}$	In house
IL-17Ra$^{-/-}$	C57BL/6Il17ra$^{-/-}$	Genetically modified on *C57BL/6* background, lacking *Il17ra* gene	Amgen Inc. (CA, US)

3.10. Transfer model of colitis

Naïve T cells from wild type, *IL-17A*$^{-/-}$ and *IL-17Ra*$^{-/-}$ C57BL/6 donor mice were adoptively transferred into *Rag1*$^{-/-}$ and *RaW* C57BL/6 recipient mice. Firstly, spleens of donor mice were dissected and crushed in PBS. Red blood cells were lysed in ACK lysing buffer (1 x PBS containing 155mM NH_4Cl, 10mM $KHCO_3$ and 1.0mM EDTA at pH 7.4) for 5 min on ice. After washing with PBS twice, single cell suspensions were obtained and counted under the microscope. More than 95% cell viability was confirmed by Trypan blue exclusion assay. $CD4^+$ T cells were enriched using the mouse $CD4^+$ T Cell Isolation Kit (Miltenyi Biotech, Germany) according to the manufacturer's instruction. Briefly, single cells were labeled with a cocktail of biotin-conjugated antibodies against non-CD4 antigens. Cells were then labeled with anti-biotin magnetic micro beads. The bead-bound non-CD4 cells were depleted by retaining on a MACS® Column (Miltenyi Biotech) in the magnetic field of an Auto MACS Separator (Miltenyi Biotech), while the unlabeled $CD4^+$ cells were enriched through the column. Isolated $CD4^+$ T cells were then stained and sorted on a FACS ARIA flow cytometer (BD Biosciences, CA, US) to obtain a pure $CD4^+CD62L^{hi}$ naive T cell population. Naive T cells (0.5×10^6/mouse) were transferred i.p. into *Rag1*$^{-/-}$ and *RaW* recipients. Bodyweight and behavior of mice were closely monitored after transfer. Mice were sacrificed to assess histological inflammation due to ethical requirements if they reached 80% or less of their original body weight.

3.11. Antibody, cytokine and drug treatments in mice

Antibodies, cytokines and drugs used are listed in Table 3.2. All reagents were reconstituted in sterile PBS. The dose and route of administration were chosen based on previous studies in experimental inflammatory diseases in mice. An isotype antibody or an irrelevant protein molecule was included in each experiment as a control, with the same concentration and delivery route.

Table 3.2 Antibody, cytokine and drug information for mouse experiments

Name	Concentration	Delivery route	Source
anti-IL-17A	0.5 mg/mouse	i.p. weekly	A gift from Amgen Inc.
anti-IL-17F	0.5 mg/mouse	i.p. weekly	A gift from Amgen Inc.
anti-IL-17E	0.5 mg/mouse	i.p. weekly	A gift from Amgen Inc.
anti-IL17Ra	0.5 mg/mouse	i.p. weekly	A gift from Amgen Inc.

anti-p19	3 mg/kg weight	s.c. biweekly	A gift from Eli Lilly
anti-p40	10 mg/kg weight	s.c. biweekly	A gift from Eli Lilly

3.12. Mouse tissue end-point collection

At the end of each experiment, tissues and organs were collected for assessment of intestinal inflammation. mLNs were collected for leukocyte stimulation and cytokine analysis. The whole colon, the caecum and ileum (distal 1/3 part of the small intestine) were removed from the mouse. Each segment of the intestine was opened longitudinally and the fecal contents were gently removed. Colon weight was measured and then the colon was dissected longitudinally into two sections. One of the sections was rolled (Swiss rolled) from the rectum along with cecum and ileum and fixed in 10% formalin. Swiss roll samples were used for histological examination and staining. The other section of the colon was dissected in equal halves, designated as ‘proximal’ and ‘distal’ colon and snap frozen in dry ice for later protein and RNA analysis.

3.13. Nucleic acid extraction and synthesis

Nucleic acid from tissues and cells were extracted as described below. Samples from individual experiments were processed at the same time in order to minimize batch to batch variation. Total yield and purity of the extracted nucleic acid was measured by the NANODROP1000 (NanoThermo,Thermo Fisher Scientific, MA, USA). The nucleic acid quantity was expressed as ng per µL and the purity was valuated based on the A260/280 ratio. The extracted nucleic acids were kept at -70°C for long-term storage.

3.14. Genomic DNA extraction

Genomic DNA was extracted from mouse blood and tissue samples using Qiagen DNeasy Blood & Tissue Kit following the manufacturer’s instructions. Around 180 µl of mouse blood samples were collected into 1.5 ml tubes with pre added 20 µl of Protease K. Samples were thoroughly mixed by vortexing after adding 200 µl of buffer AL and incubated at 56°C for 10 min. 200 µl of ethanol (96–100%) was added to samples followed by vortexing. The sample mixtures were then loaded into the DNeasy Mini spin column. After washing the membrane with 500 µL washing buffer AW1 twice, DNA was eluted in buffer AE by centrifugation. From 100 µl of blood, 3 – 6 µg of genomic DNA was usually isolated. Mouse tail tip samples usually yielded 10 – 20 µg of DNA.

3.15. RNA extraction

Total RNA was extracted from tissue and cell samples using Trizol (Invitrogen) in combination with the High Pure RNA Isolation Kit (Roche). Firstly, up to 1 x 10^6 cells or 0.1 g of tissues were homogenized in 500 µl of Trizol reagent (Life Technologies) using a FastPrep-24 machine (MP Biomedicals, CA, US) with the lysing Matrix Beads (MP Biomedicals). 200 µl of chloroform was added into the tissue-Trizol mixture and vortexed till mixed well. The mixture was then centrifuged at 12,000 g for 20 min at 4°C. The top aqueous layer containing RNA was carefully aspirated and transferred to a new tube and three volumes of 70% ethanol was added to precipitate the RNA. The ethanol RNA mixture was transferred to Roche spin column and centrifuged. The overflow liquid was discarded and DNase treatment was performed by incubating 100 µl of DNase solution with the membrane for 20 min at room temperature. After incubation, the membrane was washed twice with RW1 wash buffer. The column was then dry spun for 2 min to clean up the membrane. To the dried column, 50 µl of RNAase free water was added and allowed to incubate for 1 min at room temperature before centrifugation. Eluted RNA was checked with NANODROP1000 for concentration and purity. From 50mg of mouse colonic tissue, around 50 to 150 µg of RNA was usually obtained.

3.16. Synthesis of complimentary DNA

Complimentary DNA (cDNA) was synthesized from total RNA using an iScript cDNA synthesis kit (Bio-Rad, CA, USA) according to the manufacturer's instruction. Briefly, 1 µg of total RNA, 4 µL of 5 x iScript reaction mix (containing oligo dT and random hexamer as primer) and 1 µl iScript reverse transcriptase were mixed and toped up to 20 µl total volume with RNAase free H_2O. The reaction program was, 25°C for 5 min, 42°C for 30 min and 85°C for 5 min. cDNA was then diluted 1 in 10 with DNAase free H_2O for polymerase chain reaction.

3.17. Polymerase Chain Reaction (PCR)

3.17.1 Designing of oligonucleotide primers

To design oligonucleotide primers for PCR reactions, the reference sequence of messenger RNA was obtained online from Genome Browser website (URL: http://genome.ucsc.edu/cgi-bin/hgGateway) and Primer 3 software v.0.4.0 (URL: http://bioinfo.ut.ee/primer3-0.4.0/primer3/input.htm) was used to design primers with exon-spanning. The primer specificity was checked using nucleotide BLAST (NCBI, US). All

primers were obtained from GeneWorks (SA, Australia). Primer annealing temperature was determined first through gradient PCR. PCR conditions were deemed optimal if only a single PCR product of the correct size was amplified. For real-time PCR, the lowest primer concentration to give ≥ 95% efficiency without formation of primer dimers was used. All primers used for quantification of gene expression and their sequences are listed in Table 3.3.

Table 3.3 Primer sequences used for quantification of gene expression by real time PCR

Genes	Primer sequences (5' - 3')	Annealing temperature (°C)	Product size (bp)
Primers for mouse genes			
Atoh1	GCTTCCTCTGGGGGTTACTC GAAGGCGACAGGTCCTTCTG	60	104
Ccl1	ACCATGAAACCCACTGCCAT GTAAGCATGCTCTTGCTGTCAA	60	86
Ccl22	CCTCTGATGCAGGTCCCTATG GCAGAGGGTGACGGATGTAG	60	75
Ccl3	CGTTCCTCAACCCCCATC TGTCAGTTCATGACTTTGTCATCAT	60	91
Ccl4	CTAACCCCGAGCAACACCAT GAGCCCATTGGTGCTGAGAA	60	101
Ccl5	ATATGGCTCGGACACCACTC GTGACAAACACGACTGCAAGA	60	123
Cxcl10	TCCTTGTCCTCCCTAGCTCA ATAACCCCTTGGGAAGATGG	60	124
Cxcl9	TGGAGTTCGAGGAACCCTAGT TTGTAGTGGATCGTGCCTCG	60	78
Gapdh	AGCCCAAGATGCCCTTCAGT GACGTGCCGCCTGGAGAAAC	60	92
Grp78	TGCTGCTAGGCCTGCTCCGA CGACCACCGTGCCCACATCC	60	257
Hes1	GGTCCTAACGCAGTGTCACC	60	120

	GAGAGGTGGGCTAGGGACTT		
Ifng	GCTTTGCAGCTCTTCCTCAT GTCACCATCCTTTTGCCAGT	60	162
Il10	AGCTCCAAGACCAAGGTGTC TCCAAGGAGTTGTTTCCGTTA	60	195
Il12b	GACCATCACTGTCAAAGAGTTTCTAGAT AGGAAAGTCTTGTTTTTGAAATTTTTTAA	60	153
Il17a	CTCCAGAAGGCCCTCAGACTAC AGCTTTCCCTCCGCATTGACACAG	60	141
Il17f	ATGTTGGGACTTGCCATTCT ACAGAAATGCCCTGGTTTTG	60	155
Il17ra	GAAAACCGCCACCGCTTAC AGTGTTTCCTCTACCCAGCAC	60	194
Il1b	GATCCACACTCTCCAG CTGCA CAACCAACAAGTGATATTCTCCATG	60	152
Il22	CCGAGGAGTCAGTGCTAAGG CATGTAGGGCTGGAACCTGT	60	106
Il23a	GTCCTAGTAGGGAGGTGTGAAGTTG AGCGGGACATATGAATCTACTAAGAGA	60	245
Il23r	TGTCACGGAGGAATCACAAG TGTGCATGTGAAGAGTTTGGA	60	132
Il6	TCAGGAAATTTGCCTATTGAAA GGAAATTGGGGTAGGAAGGA	60	196
Mip2a	ACGTGTTCCAGGACACAACA ACAAACCCTCCCCACCTAAC	60	181
Muc2	CCATTGAGTTTGGGAACATGC TTCGGCTCGGTGTTCAGAG	55	104
Spdef	AAAGCCACTTCTGCACGTTACCAG GTTGCCTGCTACTGTTCCCAGATG	59	408
sXbp1	GAGTCCGCAGCAGGTGC CAAAAGGATATCAGACTCAGAATCTGAA	60	102

Tbp *(*Tata box binding protein*)*	GGGAGAATCATGGACCAGAA CCGTAAGGCATCATTGGACT	60	113
Primers for human genes			
ATOH1	AACGCCTTGTCCGAGCTGCTA TTTTGCAGGAGGCTGGAG	60	73
GAPDH	CCTGTACGCCAACACAGTGC ATACTCCTGCTTGCTGATCC	60	211
GRP78	GCCTGTATTTCTAGACCTGCC TTCATCTTGCCAGCCAGTTG	60	150
HES1	AGTGAAGCACCTCCGGAA TCACCTCGTTCATGCACTC	43	113
IL8	TGCAGCTCTGTGTGAAGGTGCAG TGTGTTGGCGCAGTGTGGTCC	60	145
LGR5	TCACCTTCCCCAGGCCCCTT CGCATCCACACCAGCCAGCA	60	231
MUC2	CAGCACCCATTGCTGAGTTG GCTGGTCATCTCAATGGCAG	55	140
SPDEF	GCACTGCAGCAGACA GGGGATACGCTGCTC	59	105
sXBP1	GAGTCCGCAGCAGGTGC CAAAAGGATATCAGACTCAGAATCTGAA	60	102

3.17.2 Quantification of gene expression by SYBR Green real-time PCR

Gene expression levels were determined by quantitative real-time PCR in 384-well plate format using SYBR Premix Ex Taq (Takara, Shiga, Japan) and Applied Biosystems® HT7900 or ViiA™ 7 Real-Time PCR Systems (Life Technologies). The total PCR reaction volume is 7.5 µL and master mix contents, and concentrations of each component are listed in Table 3.4. qPCR thermocycling conditions were 40 cycles amplification including 15 s at 95°C and 60 s at annealing temperature with an initial hold cycle for 10 min at 95°C. The selected annealing temperatures of individual primers are listed in Table 3.3.

Table 3.4 qPCR reaction master mix contents.

Reagent	Volume	Final Concentration
SYBR® Premix Ex Taq II (2×)	3.75 µl	1 ×
Forward Primer (2 µM)	0.75 µl	0.2 µM
Reverse Primer (2 µM)	0.75 µl	0.2 µM
ROX Reference Dye (50 ×)	0.15 µl	1 ×
Template (< 100 ng)	2.5 µl	−
dH_2O (sterile distilled water)	0.35 µl	−
Total	7.5 µl	−

3.18. Histological staining, morphometry and assessment of colitis

3.18.1 H&E staining

Formalin fixed tissue samples were processed into paraffin blocks and sections with the help of QIMR Histology Service and TRI Histology Service. After de-waxing in Xylene and re-hydrating through ethanol gradients (100%, 95%, 90% 70% and dH_2O), paraffin sections were placed in Haematoxylin reagent (Gill's formula, No.2, Sigma-Aldrich) for 30 s and then washed under running tap water for 5 min. Slides were then placed in Eosin Y reagent (Sigma-Aldrich) for 30 s. After Eosin staining, slides were dehydrated in 90%, 100% ethanol and xylene, followed by mounting with Pertex® mounting medium (Medite, Burgdorf, Germany) and examination under a bright field microscope for histology.

3.18.2 Immunohistochemistry

Paraffin embedded tissue sections were de-waxed in xylene, re-hydrated through ethanol gradients (100%, 90% and 70%) and placed in PBS. Different antigen retrieval methods were used for different antigens. For Muc2 staining, slides were boiled in citric acid buffer (10 mM citric acid and 0.05% Tween-20, pH 6.0) for 20 min in a microwave oven. For neutrophil staining, slides were treated with Medical Diva retrieval buffer (Biocare Medical) for 15 min at 105 °C using the Biocare Medical decloaking chamber. For F4/80 staining, Carezyme I Trypsin Kit (Biocare Medical) was used according to the manufacturer's instructions. Briefly, slides were incubated with 1 in 2 diluted trypsin concentrate solution for 15 min at room temperature. After antigen retrieval steps, the endogenous hydrogen peroxidase activity was quenched by incubating slides with 3% hydrogen peroxidase in PBS for 10 min. Sections were then blocked with 10% KPL

blocking solution (KPL Inc, USA) in PBS for 1 h at room temperature. Each primary antibody was titrated first to define the optimal staining concentration. For Muc2, 1 in 500 diluted rabbit anti-mouse Muc2 antibody (Muc2.3) was incubated with slides overnight at 4°C. For neutrophil staining, rat anti-mouse Ly-6G/6C (clone: NIMP-R14, Abcam) antibody was diluted 1 in 100 in Biocare Medical Da Vinci Green and incubated with sections overnight at 4°C. For F4/80, rat anti-mouse F4/80 antibody (clone: A3-1, Abcam) was diluted 1 in 350 in PBS with 5% FCS and applied with sections for 90 min at room temperature. After incubation with primary antibody, slides were washed 3 times in PBS. HRP-conjugated secondary antibodies against the species primary antibody raised in were incubated with sections for 2 h at room temperature. Unbound antibodies were washed with PBS and signal was detected using diaminobenzidine solution (Dako, CA, US). After counter-stained with haematoxylin, sections were dehydrated ethanol gradient and Xylene before mounting with Pertex® mounting medium (Medite).

3.18.3 Immunofluorescent staining on tissue section

Immunofluorescent staining was performed on tissue sections. For tissue samples, the same procedures were followed as immunohistochemistry staining except sections were incubated with a fluorochrome conjugated secondary antibody and mounted with ProLong® Gold Antifade Reagent with DAPI (Life Technologies). Staining was examined using BX63 Motorized Upright Microscope (Olympus).

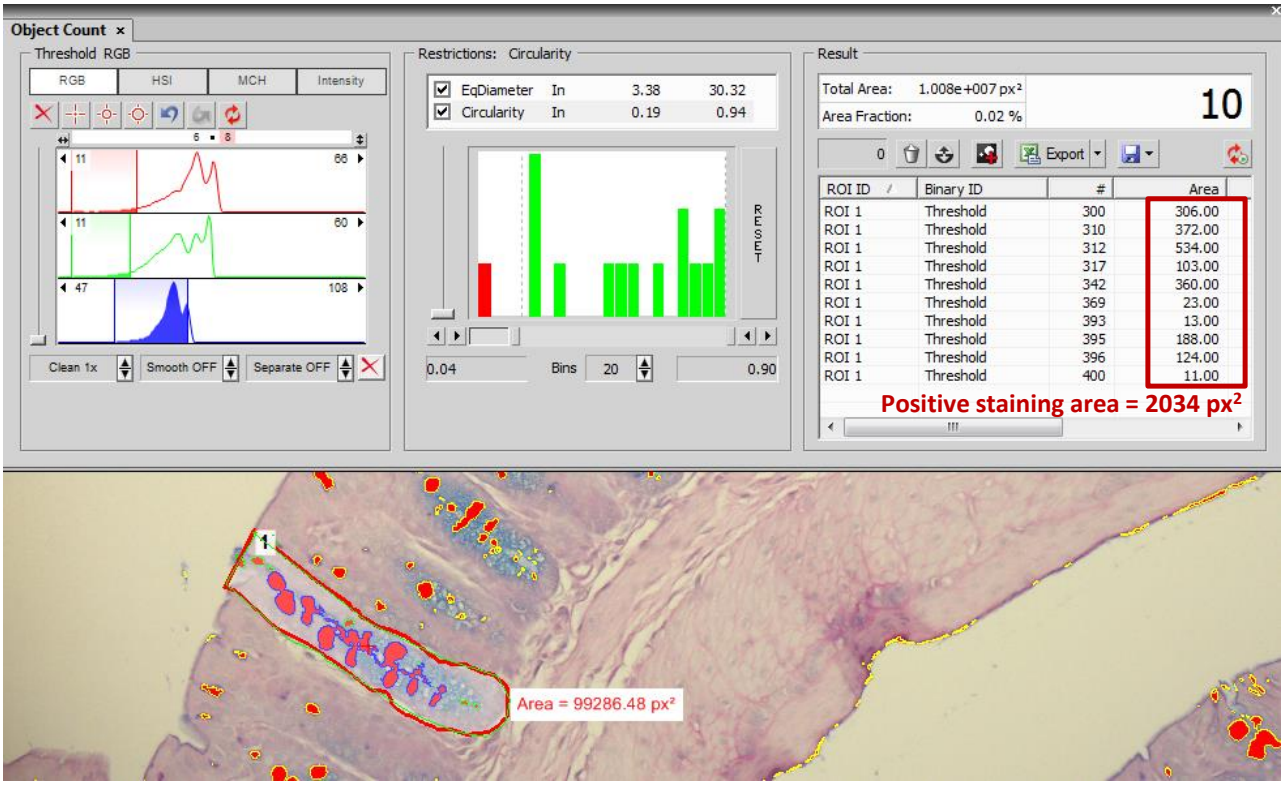
3.18.4 Alcian blue and periodic acid Schiff's (PAS) staining

Alcian blue and periodic acid Schiff's staining was used to assess the goblet cell mucin storage. After de-waxing in xylene and re-hydrating through ethanol gradients, paraffin sections were placed in Alcian blue solution (1% Alcian blue in 3% acetic acid, pH 2.5) for 10 min and washed under running tap water. Then slides were treated with 1% periodic acid for 5 min and washed with dH_2O before staining in Schiff's fuchsin-sulfite reagent (Sigma) for 10 min. Slides were washed under running tap water for 5 min and then counterstained with haematoxylin (Gill's Formula No.2, Sigma) for 30 s, dehydrated and mounted with Pertex® mounting medium (Medite).

3.18.5 Morphometry

To quantify positive staining in tissue sections after immunohistochemistry and histochemistry staining, multiple pictures from areas with longitudinally sectioned crypts

were taken randomly using an Olympus bright field microscope with a 40× lens in order to keep sampling process unbiased. In each image, positive staining was defined by RGB pixel picking tool in NIS-Elements software v.3.0 (Nikon, Tokyo, Japan). The percentage of the longitudinally sectioned crypt area occupied by positive staining was calculated (Figure 3.1) for multiple crypts within each specimen and the average for that specimen determined, and used for statistical analysis.



% of positive staining per crypt = area covered by positive staining / area of the crypt

Figure 3.1 Quantification of positive staining by using NIS-Elements software. Positive staining was defined by RGB pixel picking tool in NIS-Elements software. For example, to quantify goblet cell volume in a longitudinally sectioned crypt, the percent of positive staining per crypt is calculated as the area covered by positive staining divided by the area of the crypt.

3.19. Histological scoring of colitis

H&E stained mouse intestinal sections were scored microscopically in a blinded manner by RW and MAM including assessments of crypt architecture, crypt abscesses, tissue damage, goblet cell depletion, inflammatory cell infiltration and local neutrophil counts. Crypt length was measured using cellSens software (Olympus). The maximum combined score for each part of the intestine was 25 as per a standard scoring system (Appendix B).

3.20. Mesenteric lymph node (mLN) culture

Mouse mesenteric lymph nodes were dissected from mesenteric fat. After carefully removal of the connective and adipose tissue, mLNs were crushed and filtered through a 40μM cell strainer. A single cell suspension of leukocytes were resuspended in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, 200 U/ml penicillin and 200 μg/ml streptomycin. 2 x 10^6 cells were plated in 24-well plate (1ml per well) and stimulated with anti-CD3 and anti-CD28 antibodies (eBioscience) for 48 h. Culture supernatants were collected and stored at -80°C for further analysis.

3.21. Colonic epithelial cell isolation

To prepare colonic epithelial cells, mouse colons were opened longitudinally, rinsed with PBS and then Hanks' Balanced Salt solution (HBSS) with 200 U/ml penicillin and 200 μg/ml streptomycin for four times to remove the fecal content. Washed colons were cut into approximately 1 cm pieces and digested in HBSS with 5 mM EDTA and 1 mM DTT at 37°C in a shaking water bath for 15 min. The epithelial cells were then dislodged from the muscular layer and submucosa by vigorous shaking for 20 s. The supernatant containing crypts was pooled together from sequential digestions. After settling at 1 x g for 5 min, the supernatant was discarded. Crypts containing colonic epithelial cells were washed with PBS and collected by further centrifugation at 500 x g for 5 min. The pellet containing crypts was snap frozen on dry ice then stored at -80°C for further analysis.

3.22. Cytokine quantification by enzyme linked immunosorbent assay (ELISA)

Cytokines were measured in mLN culture and cell line culture supernatants using commercially available enzyme linked immunosorbent assay (ELISA) kits. Mouse IL-17A, IL-17F and human IL-8 levels were detected by kits from R&D System, and all other cytokines were detected by kits from BD Biosciences following the manufacturer's instructions. Briefly, cytokine concentrations were quantified using double determinant assay systems with capture, detection antibody and horseradish peroxidase (HRP) conjugates. Antibody binding was visualized by adding a tetramethylbenzidine (TMB) chromagen and hydrogen peroxide substrate solution mixture. Development of color was stopped by the addition of 50 μl 1N H_2SO_4. Color absorbance was measured at 450 nm wavelength with correction at 570 nm wavelength using a iMark™ Microplate Reader (Bio-Rad, CA, US). A *4-parameter logistic* standard curve was generated with each assay using the MPM6 software v.6.1 (Bio-Rad). Samples having an absorbance value falling out of

the top range of the standard curve were diluted and re-measured. Samples having an absorbance value falling out of the bottom range of the standard curve were given the concentration of the lowest standards.

3.23. Mouse peritoneal polymorphonuclear cell (PMN) isolation

To isolate PMN from the mouse peritoneal cavity, mice were administered 1 ml of 9% (w/v) casein (sodium salt, Sigma-Aldrich) in PBS (with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$). After overnight development of the inflammatory response, the casein injection was repeated the next morning with or without recombinant IL-23 (50 µg/kg). Three hours after the second injection, mice were euthanized by carbon dioxide asphyxiation. For PMN harvest, a ventral midline incision on the mouse abdominal skin was made with scissors, the abdominal skin was retracted with forceps and the intact peritoneal wall was exposed. Five ml of sterile harvest solution (PBS with 0.02% EDTA, filter sterilized) was injected into the mouse peritoneal cavity using a 21-G needle. After gentle massage the abdomen of the mouse, peritoneal fluid with PMN was taken out via a 21-G needle and transferred to a 50 ml sterile falcon tube. The process was repeated in order to remove the remaining cells from the mouse peritoneum. Harvested cells were washed with PBS (calcium and magnesium free) and counted with viability assessment by trypan blue exclusion. PMNs were purified by Percoll density gradient centrifugation: 3-5 x 10^4 peritoneal exudate cells resuspended in 1 ml of PBS were mixed with 9 ml of Percoll gradient solution in a 10 ml Beckman ultracentrifuge tube followed by ultracentrifugation at 60,650 x g for 20 min, 4°C. PMN was collected from the second opaque layer of the tube and washed with PBS twice. PMN number and viability were checked by trypan blue dye exclusion assay. More than 95% cell purity was confirmed by Giemsa staining after each isolation.

3.24. Immunoblot analysis

LS174T cells were seeded in 6-well culture plates at 80% confluence. After overnight attachment, LS174T cells were treated with recombinant human TNF-α (50 ng/ml, R&D System) and IL-23 (50 ng/ml, R&D System) for different time points (see Figure 6.10 legend for details). Then cells were collected, washed twice with cold PBS, pH 7.4 and then lysed on ice for 45 min with RIPA cell lysate buffer (RIPA buffer: 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.0 % Nonidet P-40, 0.1% sodium deoxycholate, and 1.0% protease inhibitor cocktail) while stirring every 5 min. The resulting lysates were centrifuged at 16,000 g for 20 min at 4°C to remove cell debris. Aliquots of the cell lysates

from each treatment condition were resuspended in SDS-PAGE Laemmli buffer (0.05M Tris-HCl, pH 6.8, 1% 2-mercaptoethanol, 1.0% SDS, 5% glycerol, and 0.15 bromophenol blue), boiled for 5 min at 100°C, and resolved on 4-12% acrylamide gel. Resolved proteins were transferred to polyvinylidene difluoride (PVDF) membranes, probed with anti-β-actin (Novus Biologicals, Littleton, CO, USA), anti-NF-κBp65 (phospho-536, Cell Signaling Technology) and phospho-468, Cell Signaling Technology), anti-phospho-IκBα (Cell Signaling Technology), or anti-phospho-p38 (Cell Signaling Technology) antibodies, followed by appropriate secondary antibody labelling. Detection was performed by dual-label infra-red analysis on ODYSSEY® CLx machine (LI-COR, NE, US). Data was analyzed using Odessay v.3.0 software.

3.25. MUC2 quantification by inhibition ELISA

Supernatants (containing secreted MUC2) and cell lysates (containing intra-cellular MUC2) were collected from LS174T cells after various treatments. Samples were first reduced using 50 mM DTT and then alkylated by 0.125 M iodoacetamide before further analysis. Human MUC2 antibody (Santa Cruz Biotechnology) was used to determine the levels of intra-celullar and secreted MUC2 using an enzyme-linked immunosorbent inhibition assay we previously described (Devine et al., 1993). Briefly, purified MUC2 was coated onto assay plates overnight, wells were washed and non-specific binding blocked with 2% BSA in PBS, and standards containing MUC2 or samples diluted in 2% BSA containing diluted anti-MUC2 antibody in PBS introduced into the wells. In this inhibition assay MUC2 in standards or samples inhibits antibody binding to the MUC2 coated onto the plate. MUC2 concentrations are presented as arbitrary units/ml using standard curves derived from purified LS174T MUC2.

3.26. Statistical analysis

Statistical analyses were performed using GraphPad PRISM version 5.01 (GraphPad Software, Inc, California). To compare differences between groups, the Kolmogorov-Smirnov test was performed to confirm if values come from a normal distribution. If data appeared log-normally distributed, values were log transformed before assessing the normality of the distribution, and if so then log-transformed values were used for subsequent parametric analysis. Parametric tests (One-way ANOVA with Bonferroni's multiple comparison test) were performed after confirmation of a normal distribution, or, where a normal distribution could not be confirmed or sample size was

small, non-parametric Mann-Whitney U-tests or the Kruskall-Wallis test with a post-hoc test were performed as indicated in the figure legends. For survival analysis, the Log-rank (Mantel-Cox) test was used to compare difference between two groups.

4.0 Roles of IL-17A - IL-17Ra Axis in Experimental Models of Colitis

4.1. Overview

Although T_H17 cells are involved in the pathogenesis of several autoimmune diseases and inflammatory disorders such as psoriasis, multiple sclerosis and rheumatoid arthritis, and are generated in large amounts during intestinal inflammation (Tesmer et al., 2008), the actual role of the T_H17 effector cytokines in intestinal inflammatory pathology is less clear. Contradictory findings have been reported in different murine models of colitis. In a T cell dependent colitis model, lymphopenic *Rag1*$^{-/-}$ mice transferred with IL-17A deficient naïve T cells develop exacerbated colitis compared to recipients of wild-type (WT) T cells. Increased IFN-γ levels were found in the intestinal mucosa of mice lacking T cell-derived IL-17, which indicated that IL-17A cytokine production by T cells exerts protection by antagonizing production of another pro-inflammatory cytokine, IFN-γ (O'Connor Jr et al., 2009). In an acute experimental colitis model, *IL-17A*$^{-/-}$ mice had exacerbated DSS-colitis compared to WT (Yang et al., 2008a). However, another T cell transfer study showed that neither IL-17A nor IL-17F is necessary for T cell transfer-induced colitis (Izcue et al., 2008). Therefore, the physiological role of the IL-17A - IL-17Ra pathway in intestinal inflammation and colitis progression needs further examination.

The T cell transfer model of colitis has been extensively studied and widely employed as an experimental colitis model in the past decade (Powrie et al., 1993). It is the best-characterized model of colitis induced by disruption of T cell homeostasis. In this model, donor naive T cells are transferred into a severe lymphopenic host such as SCID or Rag1/2-deficient mice. In the absence of regulatory T cells and anti-inflammatory cytokines, donor naïve T cells induce severe colonic and small intestinal inflammation with the time post-transfer to develop colitis dependent on the microbial flora in the animal facility. Histopathological changes in the distal colon include mucosal inflammation, epithelial cell hyperplasia, goblet cell depletion, polymorphonuclear and mononuclear cell infiltration, crypt abscesses and epithelial erosion. Recipient mice exhibit varying degrees of diarrhoea, rectal bleeding, and body weight loss depending on the strains of donor and recipient mice (Powrie et al., 1993). The significant advantage of this model is that T cell-mediated immune responses and inflammation can be examined (Ostanin et al., 2009). At the same time, T cell transfer colitis develops in a lymphopenic host, which does not reflect

the nature and complexity of human colitis. Thus findings from this model need to be confirmed with other relevant experimental colitis models.

In mice a wide range of genetic defects (gene knock-out or mutations) develop spontaneous intestinal inflammation. These models are useful when studying the effects of gene deficiency in the development of colitis and intestinal inflammation. Unlike T cell transfer induced colitis, murine models of genetic deficiency allow study of the roles of cytokines from a more systemic perspective.

In this chapter, to evaluate the role of the IL-17A - IL-17Ra axis in T cells, the naïve T cell-induced transfer colitis model was used. IL-17A$^{-/-}$ or IL-17Ra$^{-/-}$ T cells were transferred into immunodeficient recipient *Rag1*$^{-/-}$ mice. *Rag1*$^{-/-}$ recipients with an epithelial defect arising from *Winnie* allele were included to examine the contribution of a defect in intestinal epithelial integrity to development of colitis. To investigate the physiological role of IL-17A in intestinal inflammatory pathology, *IL-17A*$^{-/-}$*Winnie* mice were generated by crossing *IL-17A*$^{-/-}$ mice with *Winnie* mice. Intestinal inflammation of mice was assessed at 8 weeks and 14 weeks of age to study the contribution of IL-17A to the development and progression of colitis.

4.2. Methods

4.2.1 Naïve T cell transfer model of colitis

Purified naïve T cells were transferred into 6 week old *Rag1*$^{-/-}$ or *RaW* mice (*Rag1*$^{-/-}$ carrying the *Winnie* mutation in *Muc2*, see 3.10 for details). Colitis development was closely monitored by bodyweight measurement and observation of behavior. Mice were sacrificed on ethical grounds when bodyweight loss was more than 20% of their initial weight. The experiment was stopped when any whole group of mice had been sacrificed due to colitis or otherwise at 12-14 weeks post-transfer (Figure 4.1).

4.2.2 Generation of IL-17A-/-Winnie mice

IL-17A$^{-/-}$*Winnie* mice were generated by cross breeding *IL-17A*$^{-/-}$ mice and *Winnie* mice. Blood or tissue samples were obtained from their litters to isolate genomic DNA and determine the genotype. Both *Winnie* and *Il17a* alleles were genotyped by PCR and only litters with the right genotype were selected to breed.

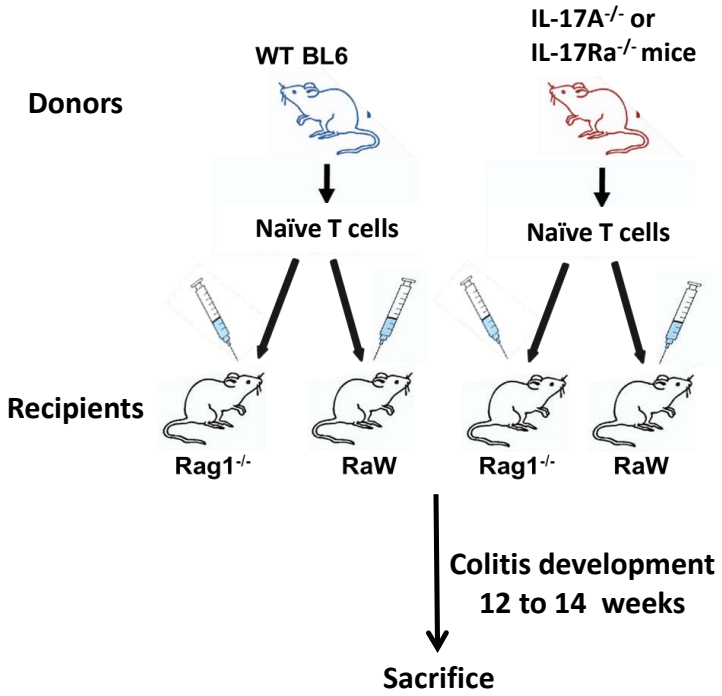


Figure 4.1 Experiment outline of the transfer model of colitis

Winnie allele genotyping – allelic discrimination assay

To genotype the *Winnie* allele, primers and Taqman probes against the single-nucleotide polymorphism (SNP) in *Muc2* gene were designed (Table 4.1, Applied Biosystems, Life Technologies). PCR reaction constituents are listed in Table 4.2, and conditions are summarized in Figure 4.2 A. Results were analyzed using Rotor Gene 6000 software (Qiagen, Australia). Basically, the reaction threshold was set to 0.1 to determine amplified or non-amplified reactions. Samples that yielded a product in FAM-Green channel, but no product in the VIC-Yellow channel were genotyped as *Winnie*. Samples that yielded a product in the VIC-Yellow channel, but no reaction in the FAM-Green channel were genotyped as *wild type*. Samples producing products in both channels were defined as *Winnie*$^{+/-}$ (Figure 4.2 B, C).

Table 4.1 Primer and probe information of *Winnie* allelic discrimination assay

SNP name	Sequences
Forward primer	TCTTCCCAAGCTGGAGACTCTT
Reverse primer	TGAAGTCATTTTTTGGTCTGGTCATCA
Wild type allele probe - VIC (Yellow)	AGGGCACTATATGTGGTCT
Winnie allele probe - FAM (Green)	AGGGCACTATATATGGTCT

Table 4.2 Master Mix contents of *Winnie* allelic discrimination assay

Reagents	Volume for 1 reaction (µL)	Final Concentration
2 x TaqMan universal PCR master mix	5	1 x
20 x working stock of SNP	0.5	1 x
DNase-free H_2O	3.5	–
DNA template (10 ng/µl)	1	1 ng/µl
Total	10	–

A

Stage	Condition
Hold	95°c for 10 min
Cycling (40 repeats)	92°c for 15 s
	60°c for 60 s, acquiring to Cycling A Green and Yellow
Melt	65 to 90°C, acquiring to Melt A Green and Yellow

B

Colour	Name	Genotype	Cycling A Green	Cycling A Yellow
	Winnie	Mutant	Reaction	No Reaction
	Winnie	Mutant	Reaction	No Reaction
	Winnie+/-	Heterozygous	Reaction	Reaction
	Winnie+/-	Heterozygous	Reaction	Reaction
	Wildtype	Wild Type	No Reaction	Reaction
	Wildtype	Wild Type	No Reaction	Reaction

C

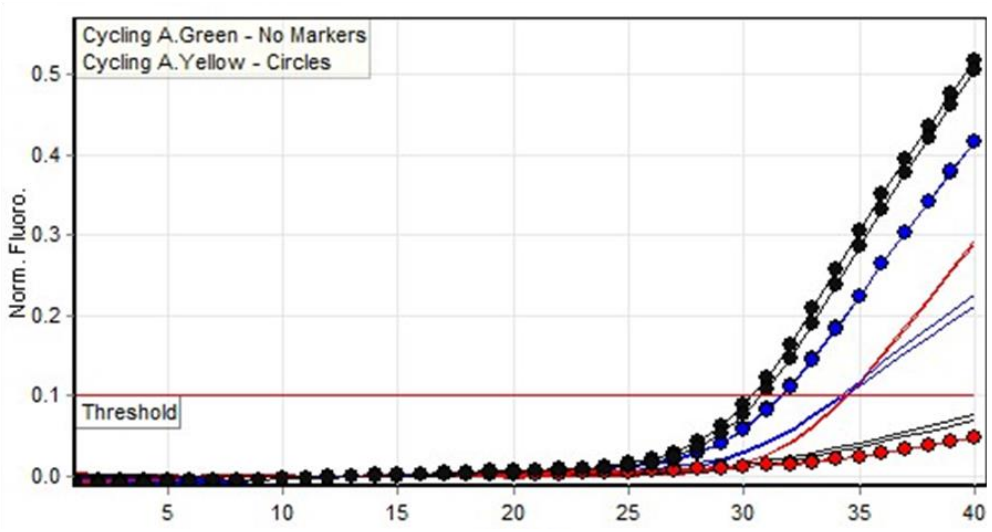


Figure 4.2 *Winnie* allele genotyping – allelic discrimination assay. (A) Assay program on Rotor-Gene 6000. (B) Allelic discrimination assay results on *Winnie*, *Winnie*$^{+/-}$ and *wild type* mice control samples (C) Gene amplification plot for 40 PCR cycles. Threshold set to 0.1 to differentiate amplified vs non-amplified reactions.

Il17a allele genotyping

To genotype the *Il17a* allele, primers of the *Il17a* allele were designed to amplify the inserted Scgb1 sequence which yields a bigger product. Primers were designed using Primer3 software and obtained from Geneworks. Primer sequences and PCR master mix contents are listed in Table 4.3 and Table 4.4. The PCR reaction included a hot start at 95° for 10 min, followed by 40 cycles of amplification with 15 s at 95°C, 30 s at 60°C and 30 s at 72°C, finished by final extension stage at 25°C for 10 min. PCR products were checked using 1% agarose gel, with an example of results in Figure 4.3.

Table 4.3 Primer information for *Il17a* allele genotyping

Primers	Sequences
Il17a$^{+/+}$ allele (forward primer)	TACAAGGCGCTTGTTGATTG
Il17a$^{+/+}$ allele (reverse primer)	TCATCACAGCAACCCTTCAG
Il17a$^{-/-}$ allele (forward primer)	TACAAGGCGCTTGTTGATTG
Il17a$^{-/-}$ allele (reverse primer)	TACTACCAGCAACCCTTCAG

Table 4.4 Master Mix contents of *Il17a* allele PCR

Reagents	Volume for 1 reaction (µL)	Final Concentration
SensiMix HRM™	6.25	1 x
Il17a$^{+/+}$ primer mix (10 µM)	1	0.8 µM
Il17a$^{-/-}$ primer mix (10 µM)	1	0.8 µM
DNase-free H_2O	2.25	–
DNA template (10 ng/µl)	2	1.6 ng/µl
Total	12.5	–

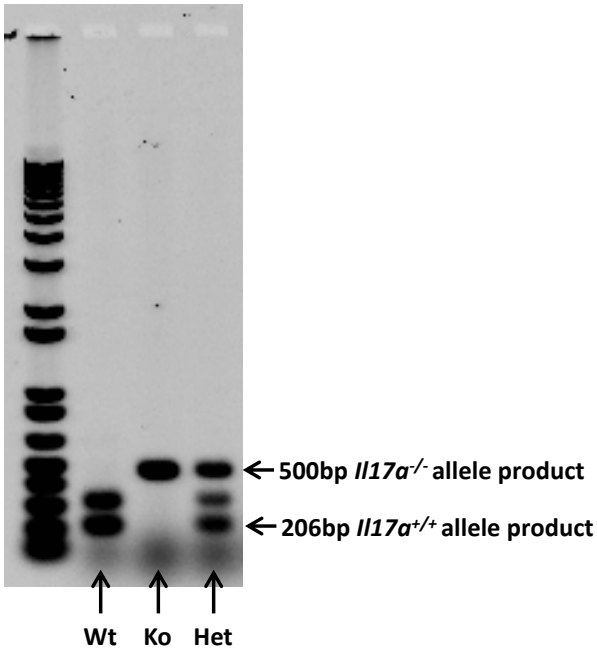


Figure 4.3 *Il17a* genotyping PCR products of wild type (Wt), heterozygous (Het) and knockout (Ko) control DNAs on 1% Agarose gel

4.2.3 Characterization of IL-17A$^{-/-}$Winnie mice

It is known that *Winnie* colitis progresses while aging. In order to examine the role of IL-17A cytokine in colitis initiation and progression, 8 and 16 week old C57BL/6, *IL-17A*$^{-/-}$, *Winnie* and *IL-17A*$^{-/-}$*Winnie* mice were sacrificed to examine the intestinal inflammation. Mice colon, caecum and terminal ilium were dissected and colon weight was measured (see 3.10 for details). Proximal and distal colons were dissected accordingly for histological colitis examination (see 3.19 for details). Proinflammatory cytokine gene expression was measured in both proximal and distal colon tissue samples by qRT-PCR. Gene expression was corrected to each sample's housekeeping gene first before being normalized to the average of the control group (see 3.17.2 for details).

4.3. Results

4.3.1 Mice with an epithelial defect develop more severe transfer colitis but IL-17A deficient and wild type T cells are equally colitogenic

We have previously reported that the progression of *Winnie* colitis is associated with T lymphocyte expansion and activation in the mLNs, especially T_H17 cells. This was confirmed and characterized by using T and B lymphocyte deficient *Rag*$^{-/-}$ mice carrying *Winnie* alleles (*RaW* mice, see chapter 1.4.4 for details) (Eri et al., 2011). Here, in order to

use *Rag*$^{-/-}$ and *RaW* mice as transfer colitis recipients and further investigate the role of naïve T cells in driving transfer colitis development, we monitored the body weight change of *Rag*$^{-/-}$ and *RaW* mice over the time period of 14 weeks starting from 6 weeks of age as well as the histological colitis at the end of monitoring. Both naïve *Rag*$^{-/-}$ and *RaW* mice showed progressively increasing body weight over the 14 weeks (Figure 4.4 A). However, on sampling at 20 weeks of age, *RaW* mice had higher colon weights and histological colitis scores in proximal and distal colon than *Rag*$^{-/-}$ mice (Figure 4.4 B,C). Elevated spontaneous colitis in *RaW* mice suggests that epithelial defect initiates mild innate local intestinal inflammation that develops in the absence of lymphocytes.

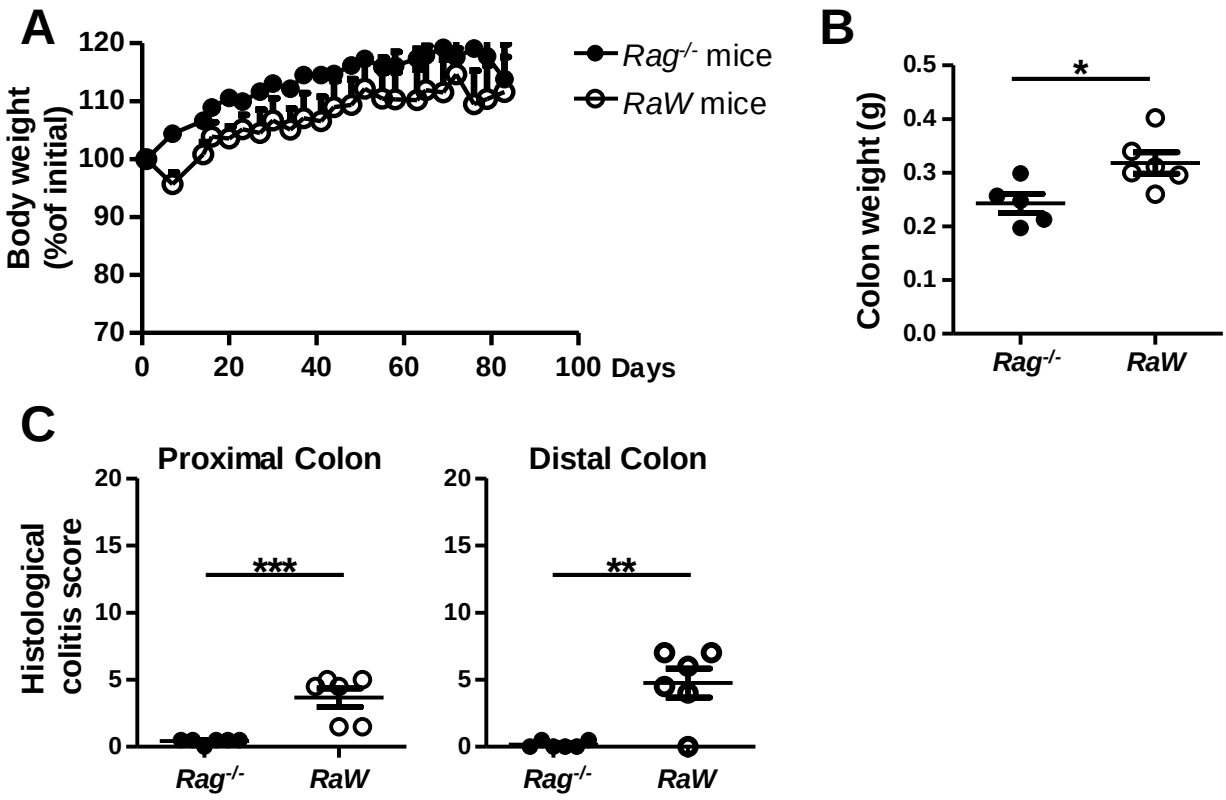


Figure 4.4 *RaW* mice develop mild spontaneous colitis. (A) Body weight monitoring over 14 weeks starting from 6 weeks (percentage of initial weight in *Rag1*$^{-/-}$ and *RaW* mice). (B) Colon weight, and (C) Histological colitis score at 20 weeks of age. Statistics: Mean ± SEM, n=6, mixed gender, single experiment; B and C: One-way ANOVA with Bonferroni's multiple comparison tests, (*P< 0.05; **P< 0.01; ***P< 0.001). These mice were the same cohort monitored alongside the experiment in Figure 4.6.

To determine the role of IL-17A in an epithelial defect model of intestinal inflammation, we employed the naïve T cell transfer induced colitis in both *Rag*$^{-/-}$ and *RaW* recipients. *RaW* mice transferred with WT and IL-17A$^{-/-}$ T cells had accelerated body

weight loss and decreased survival compared to *Rag1*$^{-/-}$ recipients (Figure 4.5, A, B). *RaW* mice developed more severe colitis than *Rag1*$^{-/-}$ recipients, as indicated by increased colon weight and histological colitis scores (Figure 4.6, A, B). However, within each mouse genotype, WT and IL-17A$^{-/-}$ T cells resulted in similar body weight loss, survival, colon weight and histological colitis. Collectively, these results indicate that, although the primary epithelial defect due to the *Winnie* allele drives a more rapid and severe onset of colitis, IL-17A production by donor T cells is not required to induce transfer colitis in either *Rag*$^{-/-}$ or *RaW* recipients.

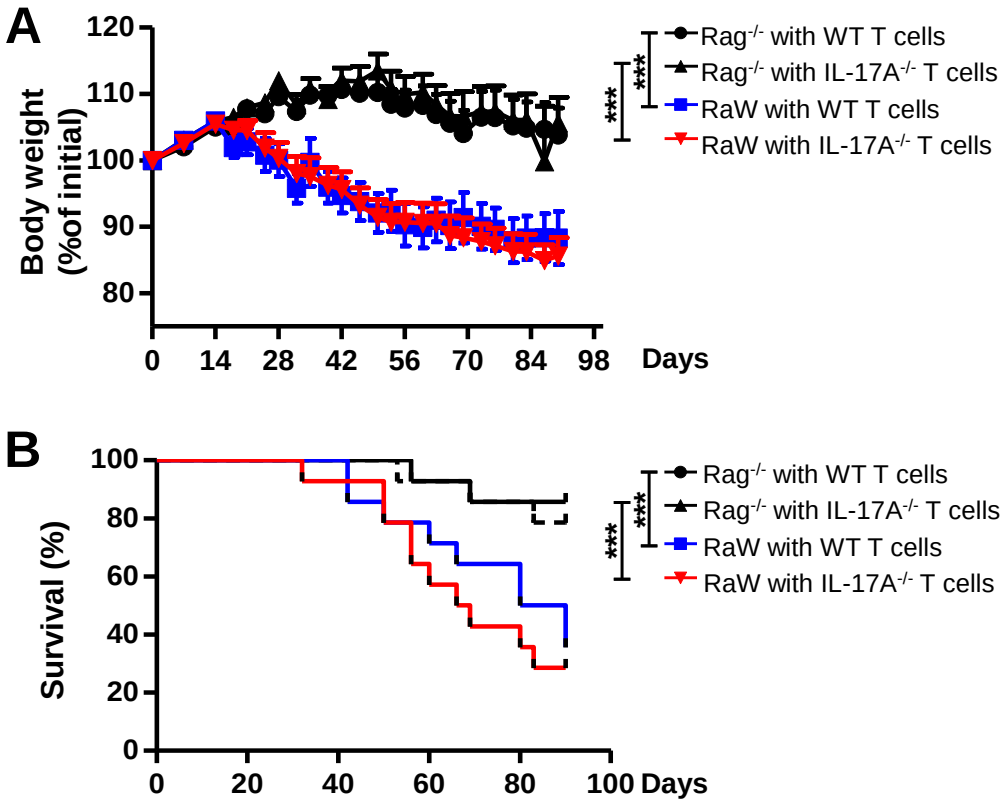


Figure 4.5 *WT* and *IL-17A*$^{-/-}$ T cells result in similar body weight loss and survival in recipient mice. *Rag1*$^{-/-}$ and *RaW* mice were transferred with naïve *WT* (*IL-17A*$^{+/+}$) or *IL-17A*$^{-/-}$ T cells. (A) Body weight (percentage of initial weight in *Rag1*$^{-/-}$ and *RaW* recipients post–transfer including mice sacrificed due to reaching 80% of their original weight). (B) Survival of mice to the ethically determined end point of 80% of original body weight. Statistics: Mean ± SEM, n=13-14, mixed gender, single experiment; A: One-way ANOVA with Bonferroni's multiple comparison tests; B: log-rank (Mentel-Cox) test (*P< 0.001).**

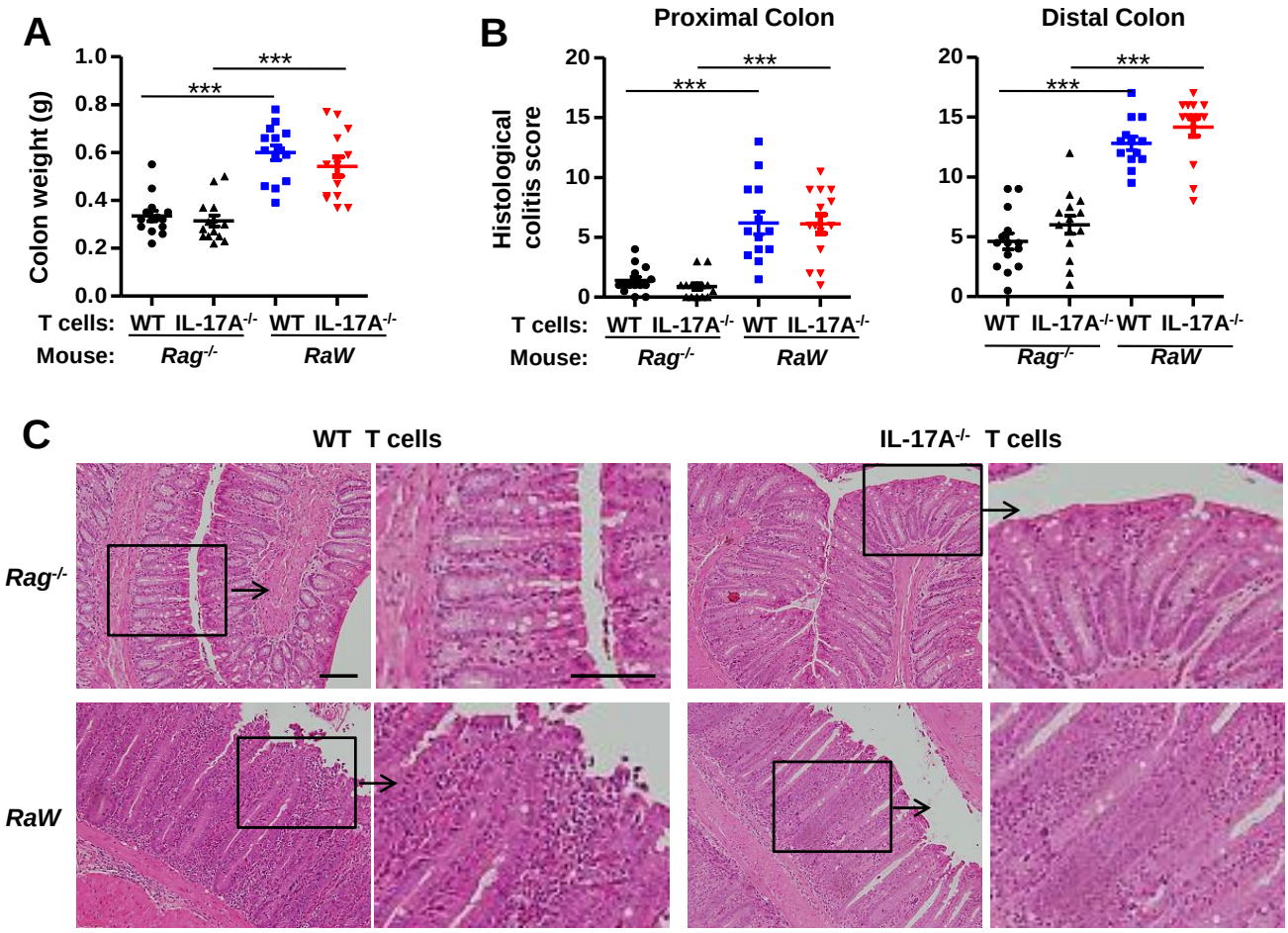


Figure 4.6 *WT* and *IL-17A*$^{-/-}$ T cells result in similar severity of histological colitis in recipient mice. *Rag1*$^{-/-}$ and *RaW* mice were transferred with naïve *WT* (*IL-17A*$^{+/+}$) or *IL-17A*$^{-/-}$ T cells. (A) Colon weight. Mice were assessed at different time points due to need to sacrifice (B) Histological colitis score. (C) Representative H&E stained sections of the distal colon. Statistics: Mean ± SEM or individual data points, n=13-14, mixed gender, single experiment; A: One-way ANOVA with Bonferroni's multiple comparison tests; B: Kruskal-Wallis test with Dunn's multiple comparison tests (*P< 0.001). Scale bars = 200 µm.**

4.3.2 IL-17 receptor signaling in donor T cells reduces the severity of transfer colitis in both Rag1$^{-/-}$ *and RaW recipients*

It is known that IL-17A cytokine signals through the IL-17 receptor (IL-17R) which is a heterodimeric complex consisting of IL17Ra and IL17Rc. To determine the importance of IL-17A responsiveness of T cells, IL-17Ra deficient T cells were transferred into lymphopenic hosts. Similarly to the previous IL-17A$^{-/-}$ T cell transfer experiment, *RaW* mice with the *Winnie* epithelial defect receiving WT and IL-17Ra$^{-/-}$ T cells had accelerated body

weight loss and reduced survival compared to the *Rag1*$^{-/-}$ recipients transferred with WT and IL-17Ra$^{-/-}$ T cells, respectively (Figure 4.7 A, B). However, unlike the situation with IL-17A$^{-/-}$ cells, within each strain, IL-17Ra$^{-/-}$ T cells induced more severe colitis than WT T cells. Loss of body weight was greater in both *Rag1*$^{-/-}$ and *RaW* transferred with *IL-17Ra*$^{-/-}$ T cells compared to mice receiving WT T cells (Figure 4.7 A). Reduced survival was also observed in both *Rag1*$^{-/-}$ and *RaW* mice receiving IL-17Ra$^{-/-}$ T cells compared to mice receiving WT T cells (Figure 4.7 B).

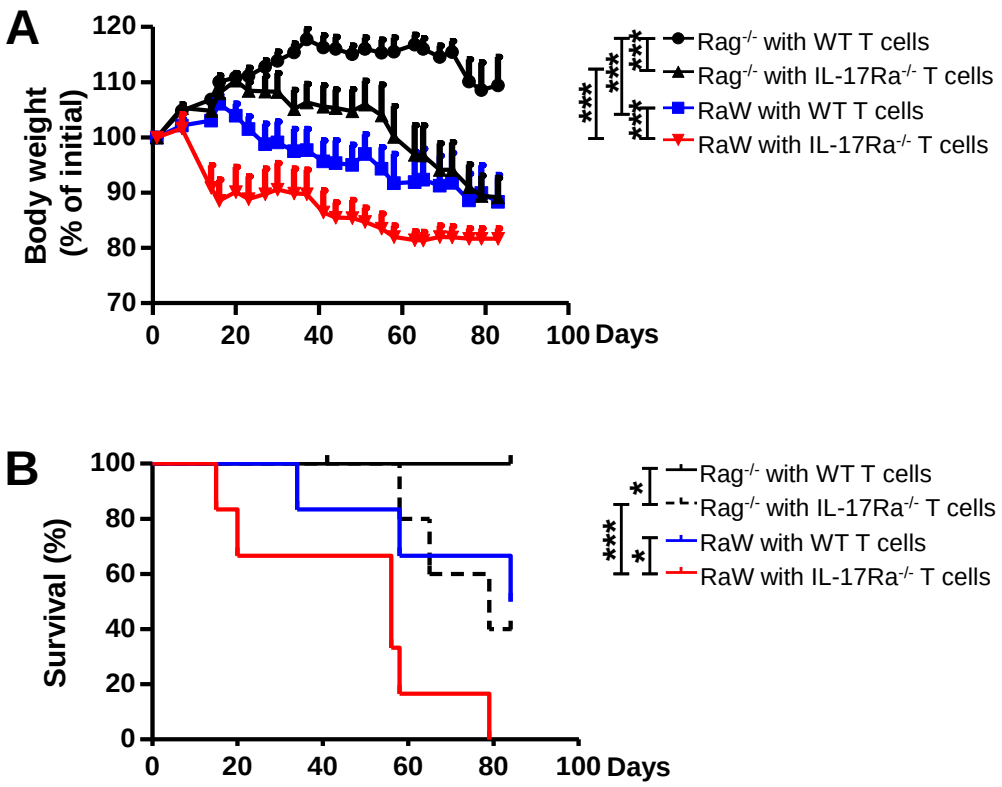


Figure 4.7 *IL-17Ra*$^{-/-}$ T cells transfer results in greater body weight loss and lower survival in recipients compared to *WT* T cells. *Rag1*$^{-/-}$ and RaW mice were transferred with naïve *WT* (*IL-17Ra*$^{+/+}$) or *IL-17Ra*$^{-/-}$ T cells. (A) Body weight (percentage of initial weight in *Rag1*$^{-/-}$ and *RaW* recipients post–transfer including mice sacrificed due to reaching 80% of their original weight). (B) Survival of mice to the ethically determined end point of 80% of original body weight. Statistics: Mean ± SEM, n=6, male only, single experiment; A: One-way ANOVA with Bonferroni's multiple comparison tests; B: log-rank (Mentel-Cox) test (*P< 0.05, ***P< 0.001).

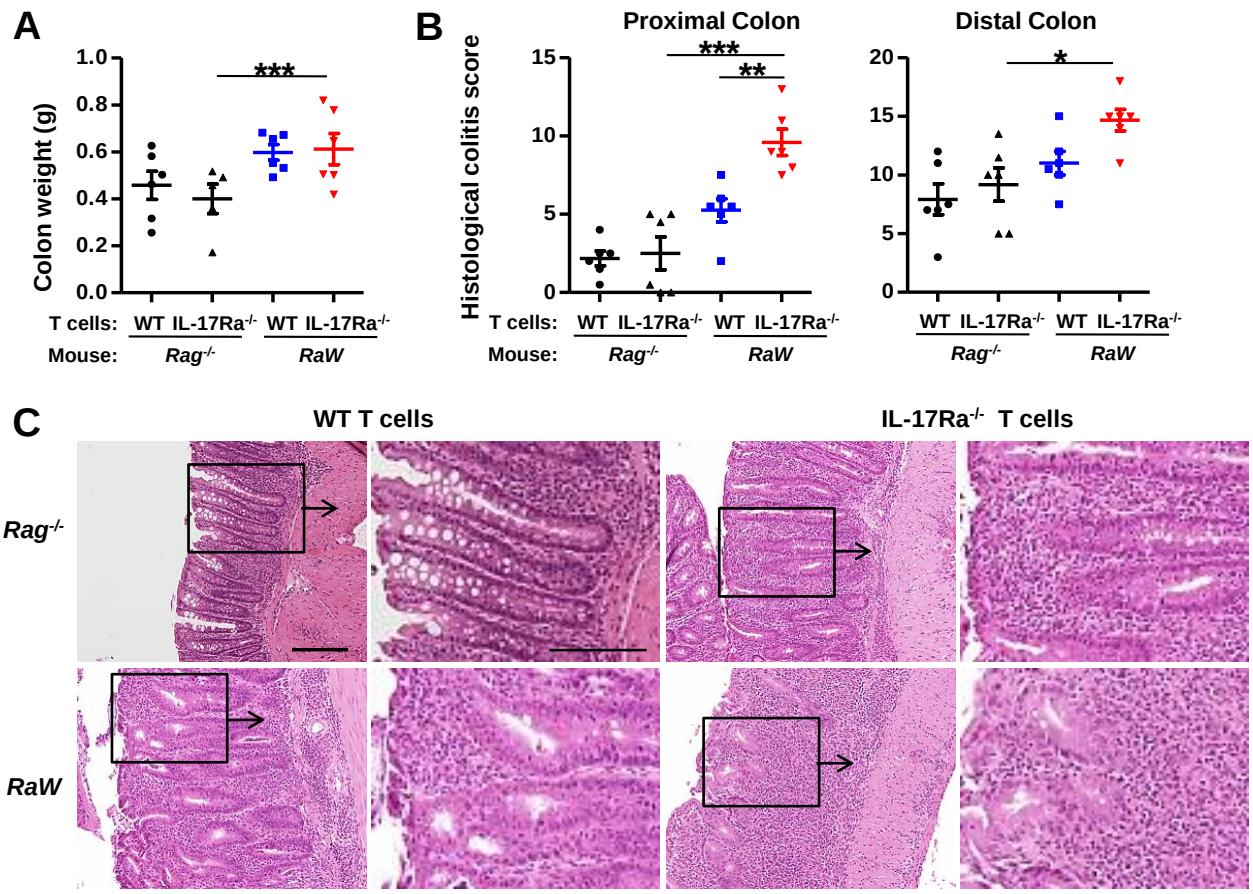


Figure 4.8 *IL-17Ra*$^{-/-}$ T cells induce more severe colitis. (A) Colon weight. (B) Histological colitis score. (C) Representative H&E stained sections of the distal colon. Statistics: Mean ± SEM and individual data points, n=6, male only, single experiment; A, B: One-way ANOVA with Bonferroni's multiple comparison tests (*P< 0.05, **P< 0.01,*P< 0.001). Scale bars = 200 µm.**

RaW mice receiving IL-17Ra$^{-/-}$ T cells also had higher colon weights and histological colitis scores than *Rag1*$^{-/-}$ mice that received IL-17Ra$^{-/-}$ T cells indicating more severe colitis (Figure 4.8 A, B). Within *RaW* mice, WT and IL-17Ra$^{-/-}$ T cell recipients had similar colon weight, but increased histological colitis was found in IL-17Ra$^{-/-}$ T cell recipients compared to WT T cell recipients (Figure 4.8 C). The score was statistically significantly increased in the proximal colon, and there was a non-significant trend in the distal colon. However, no changes in the mRNA expression of the genes encoding the proinflammatory cytokines, IFN-γ, IL-17A IL-1β, IL-6 and TNF-α, were observed between different T cell types and recipients (Figure 4.9). In summary, more severe intestinal inflammation developed in both *Rag1*$^{-/-}$ and *RaW* recipients in the T cell transfer model in the absence of IL-17Ra signaling in T cells.

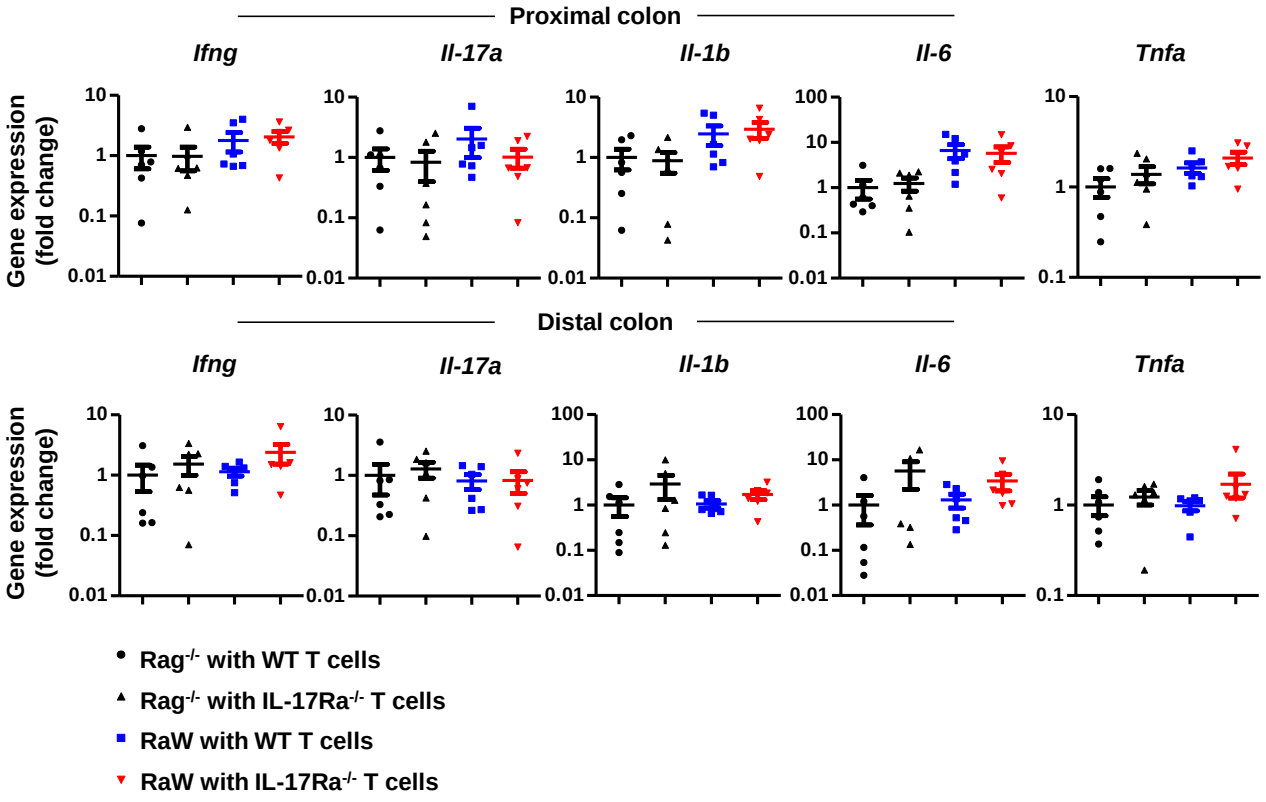


Figure 4.9 Proinflammatory cytokine gene expression did not change in *Rag1*$^{-/-}$ and *RaW* mice transferred with naïve *WT* or *IL-17Ra*$^{-/-}$ T cells. *Ifng*, *Il17a*, *Il1b*, *Il-6* and *Tnfa* mRNA expression levels in both proximal (upper panel) and distal colon (lower panel) tissue samples of *Rag1*$^{-/-}$ and *RaW* mice receiving *WT* (*IL-17Ra*$^{+/+}$) or *IL-17Ra*$^{-/-}$ T cells.

4.3.3 IL-17a deficiency does not suppress the initiation of colitis in Winnie mice

After observing that IL-17A - IL-17Ra signaling in T cells is non-pathogenic in the experimental transfer model of colitis, I decided to examine the effects of systemic deficiency of IL-17A in colitis development. Firstly, *IL-17A*$^{-/-}$ mice were cross bred with *Winnie* mice to generate the *IL-17*$^{-/-}$*Winnie* strain, which lacks IL-17A cytokine production and also has an epithelial defect. As *Winnie* spontaneous colitis exacerbates while aging, both young (8 weeks) and old (14 weeks) *IL-17*$^{-/-}$*Winnie* vs *Winnie* mice were studied to examine the contribution of IL-17A in the initiation and progression stages of colitis.

As expected, at 8 weeks of age, *Winnie* and *IL-17A*$^{-/-}$*/Winnie* mice had increased colon weight and histological colitis scores compared to age matched WT (C57BL/6) and *IL-17A*$^{-/-}$ mice. However, somewhat unexpectedly, there was no difference in these

parameters between *Winnie* and *IL-17A$^{-/-}$/Winnie* mice apart from a modest non-significant trend toward less histological colitis in the proximal but not distal colon (Figure 4.10 A, B).

Examining inflammation at the molecular level, *IL-17A$^{-/-}$/Winnie* mice had elevated *Ifng* mRNA in the proximal colon compared to *IL-17A$^{-/-}$* and *Winnie* mice (Figure 4.10 C). In contrast, in the distal colon *Ifng* expression was significantly lower in *IL-17A$^{-/-}$/Winnie* than *Winnie* mice (Figure 4.10 D). Thus IFN-γ inversely mirrors any changes in histological colitis, demonstrating that cytokine IFN-γ is not responsible for histological colitis. *IL-17A$^{-/-}$* mice had higher expression of *Il17f* than WT in both the proximal and distal colon, suggestive of a compensatory mechanism upregulating this cytokine. However, *Il17f* only trended up in the proximal colon and decreased (not significant) in the distal colon of *IL-17A$^{-/-}$/Winnie* mice (Figure 4.10 C, D). *Il1b* was elevated in the proximal and distal colon of both *Winnie* and *IL-17A$^{-/-}$Winnie* mice with no evidence of suppression with IL-17A deficiency. *Il22* gene expression was dramatically upregulated in distal colon of *Winnie* compared to WT mice, *Il17a* gene deficiency increased *Il22* gene expression in *WT* mice, but reduced *Il22* expression in the *Winnie* distal colon. *Il22* expression pattern was not altered in the proximal colon of *WT* and *Winnie* mice deficient in IL-17A (Figure 4.10 D). IL-17A deficiency changes the molecular pattern of inflammation with distal colon more protected compared to proximal colon, although histological colitis remains the same. Together this experiment suggests that genetic deficiency of IL-17A has no overall effect on the emergence of immunopathology.

4.3.4 IL-17A deficiency suppresses progression of colitis in Winnie mice

We have previously reported that intestinal inflammation in *Winnie* progresses with age (Eri et al., 2011), and therefore examined progression of colitis with IL-17A deficiency. Interestingly, given IL-17's limited effects on emergence of colitis, colitis severity did not progress with age in *IL-17A$^{-/-}$/Winnie* mice. At 14 weeks of age colon weight (Figure 4.11 A) and histological colitis scores (Figure 4.11 B) were significantly lower in *Winnie* mice deficient in IL-17A compared to *Winnie* mice. Statistical analysis has shown that the colitis level in *IL-17$^{-/-}$/Winnie* mice did not progress while aging (Figure 4.12 A,B).Histological examination revealed less aberrant crypt architecture, less goblet cell depletion, fewer crypt abscesses and less inflammatory cell infiltration, particularly in the distal colon of *IL-17A$^{-/-}$/Winnie* compared to *Winnie* mice (Figure 4.11 C). *Ifng* and *Il1b* mRNA were decreased in both the proximal and distal colon of *IL-17A$^{-/-}$/Winnie* mice compared to

Winnie, with *Il22* expression only decreased in the distal colon of *IL-17A*$^{-/-}$*/Winnie* mice (Figure 4.11 D, E). *Il17f* mRNA was increased in *IL-17A*$^{-/-}$ vs WT colons, but was similar between *IL-17A*$^{-/-}$*/Winnie* and *Winnie*. Together, this experiment shows that IL-17A contributes to the progression of *Winnie* colitis.

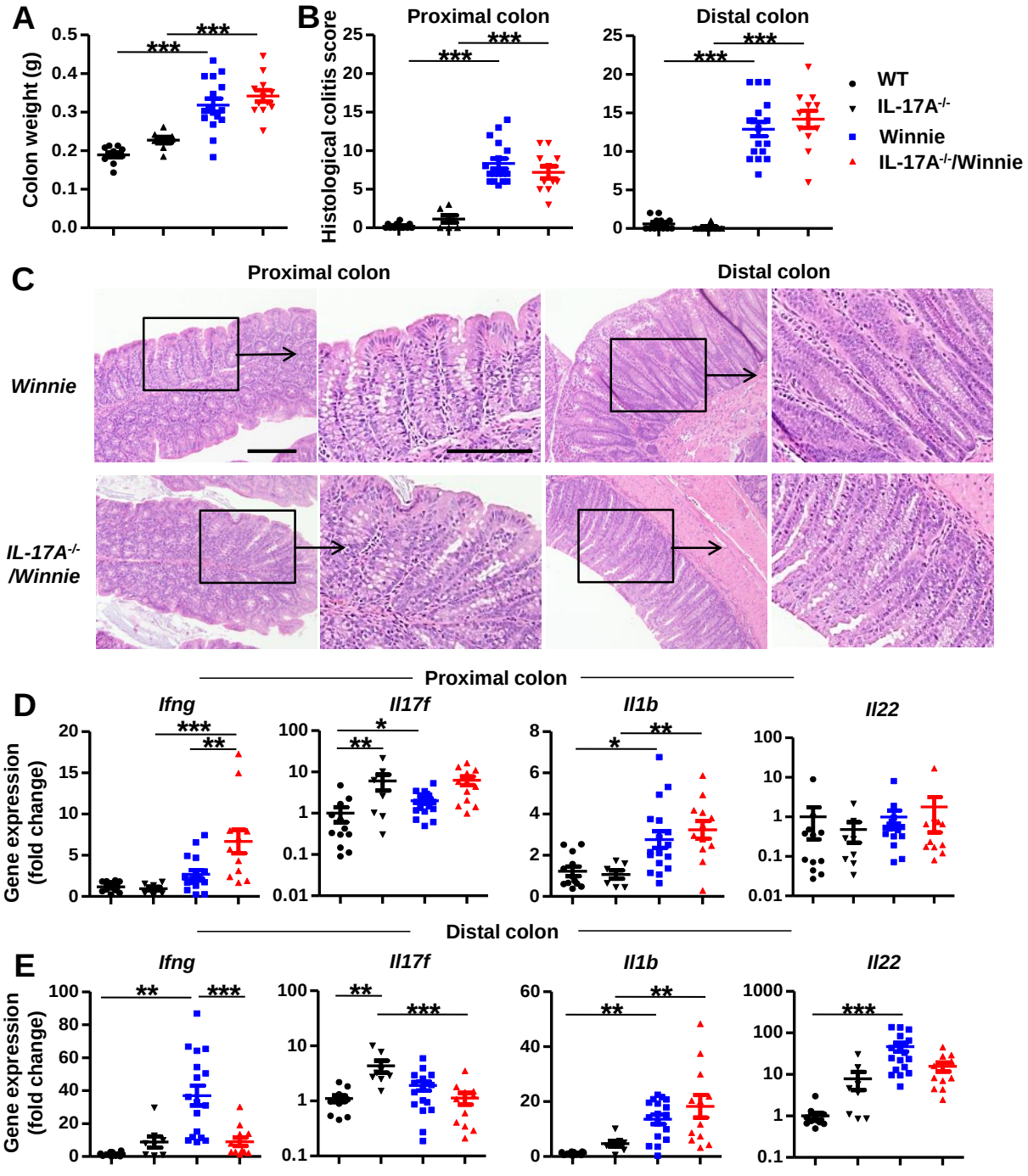


Figure 4.10 IL-17A deficiency does not modify development of colitis in *Winnie* mice. Naïve C57BL6 (WT), *IL-17A*$^{-/-}$, *Winnie* and *IL-17A*$^{-/-}$*Winnie* mice were sacrificed at 8 weeks of age. (A) Colon weight. (B) Histological colitis scores. (C) Representative H&E stained proximal and distal colon sections with an average histological colitis score from their group. (D, E) mRNA

expression levels in the proximal and distal colon. Fold change corrected to *Tata* and normalized to WT. Statistics: Mean ± SEM and individual data points, n=8-16, mixed gender, single experiment; a,c,d: One-way ANOVA with Bonferroni's multiple comparison test; b: Kruskal-Wallis test with Dunn's multiple comparison tests (P*< 0.05, ***P*< 0.01, ****P*< 0.001).**

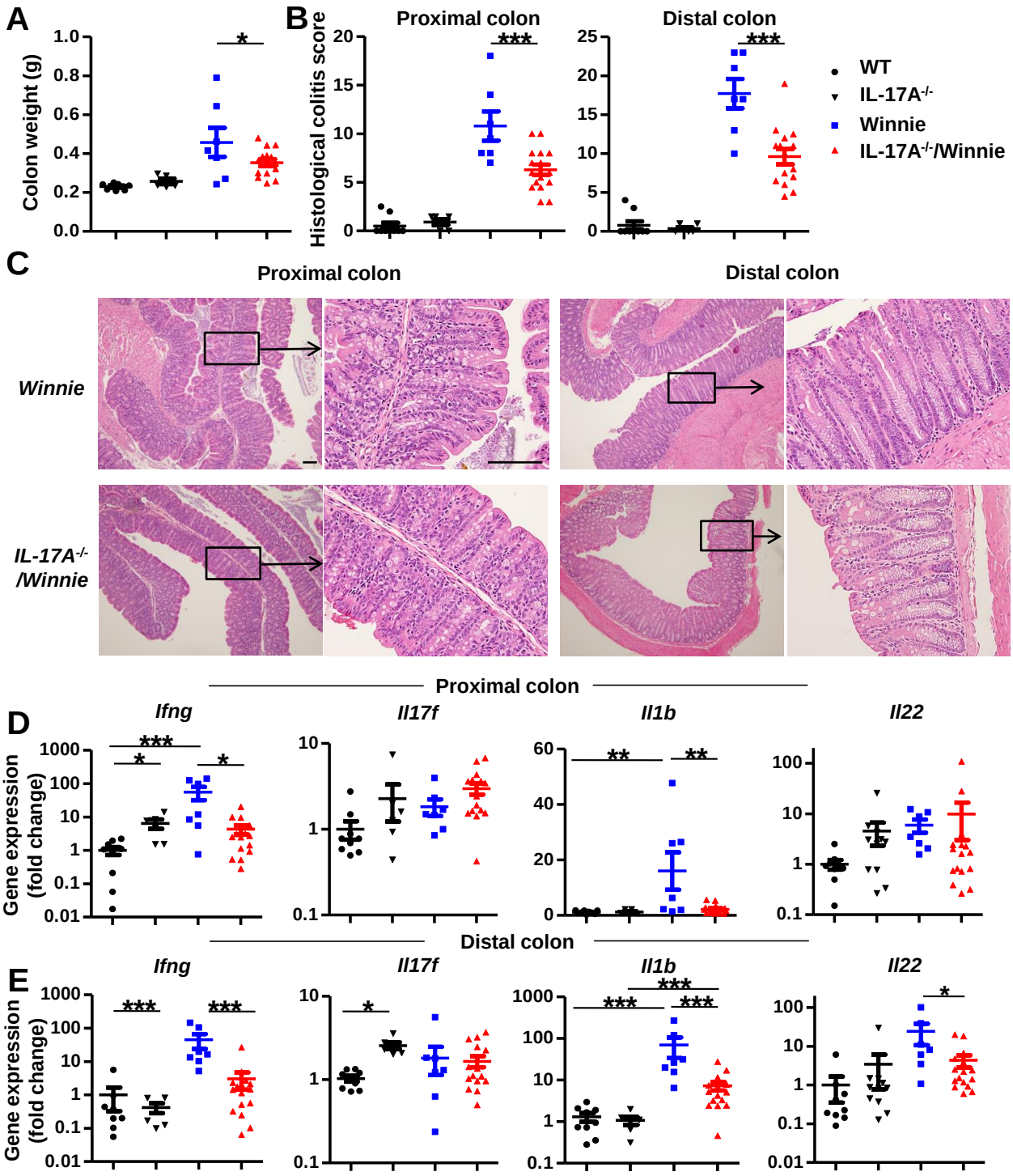


Figure 4.11 IL-17A deficiency ameliorates colitis progression in *Winnie* mice. Naïve C57BL6 (WT), *IL-17A*$^{-/-}$, *Winnie* and *IL-17A*$^{-/-}$*Winnie* mice were sacrificed at 14 weeks of age. (A) Colon weight.

(B) Histological colitis scores. (C) Representative H&E stained proximal and distal colon sections with an average histological colitis score from their group. (D, E) mRNA expression levels in the proximal and distal colon. Fold change corrected to housekeeping gene Tata and normalized to the WT group. Statistics: Mean ± SEM and individual data points, n=7-15, mixed gender, single experiment; a,d,e: One-way ANOVA with Bonferroni's multiple comparison test; b: Kruskal-Wallis test with Dunn's multiple comparison tests (*P< 0.05, **P< 0.01, ***P< 0.001). Scale bars = 100 µm.

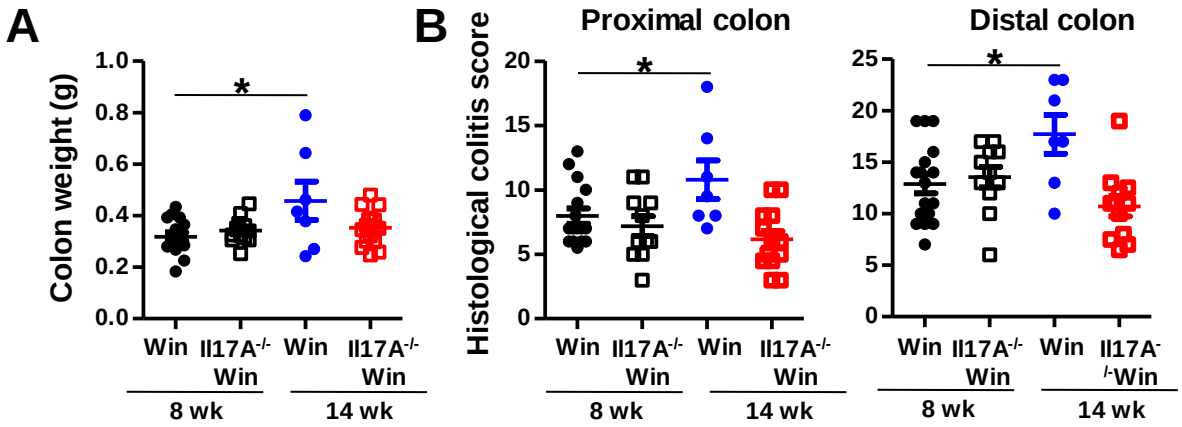


Figure 4.12 The histological colitis of *Winnie* mice deficient in IL-17A did not progress compared to *Winnie* mice. (A) Colon weight. (B) Histological colitis scores. Statistics: Mean ± SEM and individual data points, n=7-16, mixed gender; One-way ANOVA with Bonferroni's multiple comparison test (*P< 0.05).

4.4. Discussion

The first group of the experiments reported in this chapter demonstrate that T cell derived IL-17A is not required for transfer induced colitis in *Rag1*$^{-/-}$ recipients with or without an epithelial defect. These findings are consistent with previous studies showing that IL-17A signaling was not essential for the colitogenic ability of T cells (Leppkes et al., 2009). In our experiments, the inflammation induced by *IL-17A*$^{-/-}$ T cells was not due to a compensatory increase in IFN-γ production by T_H1 cells, which is supported by a previous similar study (Izcue et al., 2008). In contrast to the findings of O'Connor et al., we did not find *IL-17A*$^{-/-}$ T cells to be more colitogenic than wild type T cells in our transfer colitis experiments using either *Rag1*$^{-/-}$ and *RaW* recipients (O'Connor Jr et al., 2009). Although we concur with their findings that IL-17Ra$^{-/-}$ T cells generate more severe colitis than wild type T cells, no evidence of skewing to a T_H1 immune response was observed in our experiments. Although we used similar T cell transfer protocols, colitis was induced much

more quickly in their experiments. The cause of this discrepancy is uncertain, but may reflect differences in the intestinal microbiota between facilities, which in turn may alter the nature of the inflammation in the colon.

The difference observed between *IL-17A*$^{-/-}$ T cell and *IL-17Ra*$^{-/-}$ T cell transfer experiments was not unexpected, as IL-17A is not the only cytokine that binds to IL-17Ra. IL-17F, another effector cytokine from IL-17 family, also signals through receptor consisting IL-17Ra (Kuestner et al., 2007; Toy et al., 2006). Therefore, the pathogenic role of IL-17Ra$^{-/-}$ T cells in inducing transfer colitis could be due to the unresponsiveness of these cells to T_H17 cytokines other than IL-17A.

Impacts of genetic deficiency of IL-17A on colitis development have not been thoroughly studied. A previous study showed that *IL-17A*$^{-/-}$ mice had exacerbated DSS-colitis compared to WT controls, indicating that IL-17A has a suppressive role in chemically induced acute colitis or is involved in the wound healing process (Yang et al., 2008a). In this chapter, by using a spontaneous colitis model, *Winnie* mice, I found that IL-17A is more likely to play a role in the progression of colitis, rather than the initiation stage, as colitis did not show the characteristic increase in severity in old *IL-17*$^{-/-}$*Winnie* mice compared to age match *Winnie* mice.

Within the IL-17 family cytokines, IL-17A and IL-17F share the greatest homology. Both cytokines can form homo-dimers or IL-17A/IL-17F hetero-dimers which exert proinflammatory functions by activating tissue resident cells to secrete cytokines or chemokines. IL-17F may play a redundant role to IL-17A (Leppkes et al., 2009), but is less potent than IL-17A in recruiting neutrophils and stimulating downstream inflammatory cytokine production (Yang et al., 2008a). Results of *IL-17A*$^{-/-}$*Winnie* mice show that genetic deficiency of IL-17A results in elevated colonic IL-17F mRNA. IL-17F gene expression was also upregulated in *IL-17A*$^{-/-}$ mice lacking the *Winnie* allele, confirming that IL-17F could play a redundant role in absence of IL-17A. Interestingly, although *Il17f* gene expression increased in old *IL-17*$^{-/-}$*Winnie* mice, the progression of intestinal inflammation was attenuated, suggesting that IL-17F is probably less proinflammatory than IL-17A, at least in the absence of IL-17A.

Intestinal microflora colonization plays a critical role in the initiation and perpetuation of intestinal inflammation. Accumulating evidence demonstrates that the presence and the colonization by intestinal segmented filamentous bacteria (SFB) drives proliferation of T_H17 cells in the lamina propria of the small intestine (Atarashi et al., 2008; Ivanov et al., 2009; Lochner et al., 2011). However, how genetic deficiency of IL-17A affects the commensal microflora composition hasn't been fully characterized. Results from this chapter showing that IL-17a deficiency suppresses colitis progression could be explained by colonic microflora changes. Although *IL-17A$^{-/-}$/Winnie* and *Winnie* mice were bred from the same colony and housed under the same conditions in the same animal facility throughout the experiments to limit any impact of differences in intestinal microbial flora on colitis development, the possibility that change of microflora composition underlies the lack of progression of colitis in *IL-17A$^{-/-}$/Winnie* compared to *Winnie* mice cannot be excluded.

In summary, IL-17A - IL-17Ra signaling in T cells is non-pathogenic in transfer colitis, whereas IL-17A contributes to the progression of colitis in *Winnie* mice. The experimental colitis models used in this chapter have limitations in translating the findings into clinical therapy. Although T cell transfer colitis is a useful tool, this scenario does not reflect disease development in IBD patients under physiological conditions with immune surveillance from regulatory T cells. Also, cytokine knockout mice have immune deficiency and develop metabolic changes (Zuniga et al., 2010) making it hard to distinguish whether the assessments we have made are primary or secondary to intestinal mucosal inflammation. Therefore, cytokine targeting approaches mimic clinical therapies need to be explored. The efficacy of monoclonal antibodies against IL-17A or IL-17Ra in suppressing colitis will be investigated in the next chapter.

5.0 Assessment of the Therapeutic Efficacy of Monoclonal Antibodies against T_H17 Associated Cytokines or Cytokine Receptors in Suppressing Intestinal Inflammation in *Winnie* Mice

5.1. Overview

In the previous chapter my experiments demonstrated that IL-17 is involved in the progression of colitis in the *Winnie* model, but did not show whether therapeutic targeting of IL-17 would ameliorate pathology. To target IL-17A - IL-17Ra signaling in a clinical scenario, monoclonal antibodies against the IL-17A cytokine or IL-17A cytokine receptor have been developed and tested in several human autoimmune diseases. It has been reported that a humanized anti-IL-17A monoclonal antibody, ixekizumab, improved the clinical symptoms of psoriasis. The effects of ixekizumab occur at one week and are sustained through the 20 week treatment (Leonardi et al., 2012). In another phase two, randomized, double-blind, placebo-controlled, dose-ranging study, brodalumab, a human anti-IL-17 receptor monoclonal antibody also significantly improved plaque psoriasis after 12 weeks treatment (Papp et al., 2012). Anti-IL-17A antibody, AIN457, has also been shown effective in treating rheumatoid arthritis and chronic noninfectious uveitis (Hueber et al., 2010). These clinical trial findings demonstrate that IL-17A is involved in the pathophysiology of diverse inflammatory diseases including psoriasis, rheumatoid arthritis, and noninfectious uveitis.

In a double-blind, placebo-controlled clinical trial, patients with moderate-to-severe Crohn's disease was treated with secukinumab - a humanized anti-IL-17A monoclonal antibody. In contrast to the findings of IL-17 targeting in other inflammatory diseases, secukinumab failed to demonstrate efficacy for Crohn's disease, and blockade of IL-17A exacerbated disease in a subset of patients compared to placebo (Hueber et al., 2012). It is possible, based on mouse data, that neutralizing IL-17A promotes the production of the proinflammatory cytokine, IFN-γ, and fosters differentiation of T_H1 cells. IL-17A deficient naïve T cell transfer into Rag mice induced more severe colitis and increased intestinal IFN-γ mRNA, indicating IL-17A plays a partially protective role by antagonising pro-inflammatory T_H1 responses (O'Connor Jr et al., 2009), although my results presented in Chapter 4 failed to verify the antagonism of IFN-γ by IL-17A.

Based on results of Chapter 4 showing that genetic deficiency of *Il17a* slows the progression of colitis in *Winnie* mice, experiments in this chapter sought to evaluate the effect of IL-17 signaling in immunocompetent mice by examining the efficacy of anti-IL-17A or anti-IL-17Ra antibodies in *Winnie* mice with emerging or established colitis. Monoclonal antibodies against other T_H17 associated cytokines including IL-17F and IL-17E were also tested in the *Winnie* colitis model.

5.2. Methods

As *Winnie* colitis progresses with age, antibody treatments were carried out at two stages of colitis progression. To model the early stage of colitis development, 4 week old *Winnie* mice were treated with anti-IL-17A antibody for 4 weeks (Figure 5.1 A). To model therapy in established colitis, 12 week old *Winnie* mice were treated with anti-IL-17A and anti-IL-17Ra antibodies for 2 weeks (Figure 5.1 B). Antibody details are listed in Chapter 3.11 and treatment regimens are summarized in Table 3.2. Anti-cytokine antibodies were delivered i.p. weekly with appropriate isotype control antibodies in control mice as specified in Table 3.2. During the antibody treatment, body weight of mice was measured twice weekly (see 3.9 for details). At the end of the experiment, mice were sacrificed to examine intestinal inflammation. On the day of sacrifice, intestine tissue and mLN were collected to assess the colitis severity (see 3.12 for details). Mice colon, caecum and terminal ilium were dissected and colon weight was measured (see 3.10 for details). Proximal and distal colons were dissected accordingly for histological colitis examination (see 3.19 for details). Tissue total RNA was isolated and reverse transcribed to cDNA (see 3.15 and 3.16 for details). Expression of proinflammatory cytokine genes was measured in both proximal and distal colon tissue samples. Gene expression was corrected to each sample's housekeeping gene first before being normalized to the average of the control group (see 3.17.2 for details). Histological colitis was quantified using *Winnie* mice chronic or acute colitis scoring sheet (see 3.19 for details).

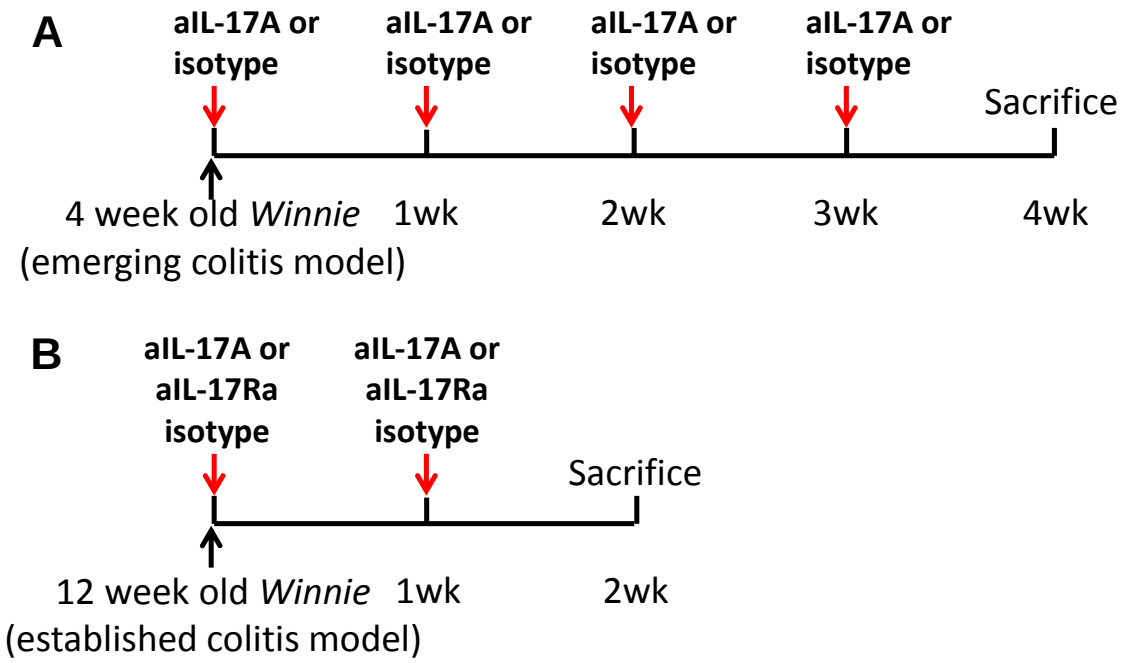


Figure 5.1 Antibody treatment regimens in C57BL6 (WT) and *Winnie* mice at different age. (A) anti-IL-17A (aIL-17A) and isotype antibody treatment of 4 week old *Winnie* mice with emerging colitis. (B) aIL-17A, anti-IL-17 receptor A (aIL-17Ra) and isotype antibody treatment of 12 week old *Winnie* mice with established colitis.

5.3. Results

5.3.1 Antibody neutralization of IL-17A promotes T_H1 immunity in Winnie mice with emerging colitis

During the 4 week antibody treatment, starting from 4 weeks of age, increases in body weight were similar in *Winnie* and *WT* mice, although both strains of mice showed increased body weight after receiving aIL-17A (Figure 5.2 A), which is similar to a previous study showing that IL-17A inhibits adipogenesis and genetic deficiency of IL-17A enhances diet-induced obesity in mice (Zuniga et al., 2010). *Winnie* mice treated with aIL-17A antibody developed more severe colitis compared to mice treated with isotype antibody as indicated by increased colon weight (Figure 5.2 B). Consistent with the increase in colon weight, aIL-17A treatment failed to ameliorate the histological colitis scores which actually trended worse in the proximal colon of antibody-treated *Winnie* mice (Figure 5.2 C). Increased inflammatory cell infiltration and tissue damage were observed in the distal colon of *Winnie* mice receiving aIL-17A antibody compare to isotype control (Figure 5.2 D).

An increase in IFN-γ production was observed in the mLN leucocytes isolated from aIL-17A treated *Winnie* mice compared to those obtained from the isotype control group (Figure 5.3 A). qPCR using distal colon tissue cDNA samples showed that *Ifng* expression in distal colon of aIL-17A treated *Winnie* mice was upregulated, but difference was not significant compared to isotype control treated group (Figure 5.3 B). The expression of T_H1-associated transcription factor T-bet was trended upward in the distal colon of aIL-17A treated *Winnie* mice (Figure 5.3 B), suggesting that neutralizing T_H17 effector cytokine IL-17A could induce T_H1 skewing in intestinal mucosa. This experiment demonstrates the importance of understanding the cytokine milieu present during intestinal inflammation.

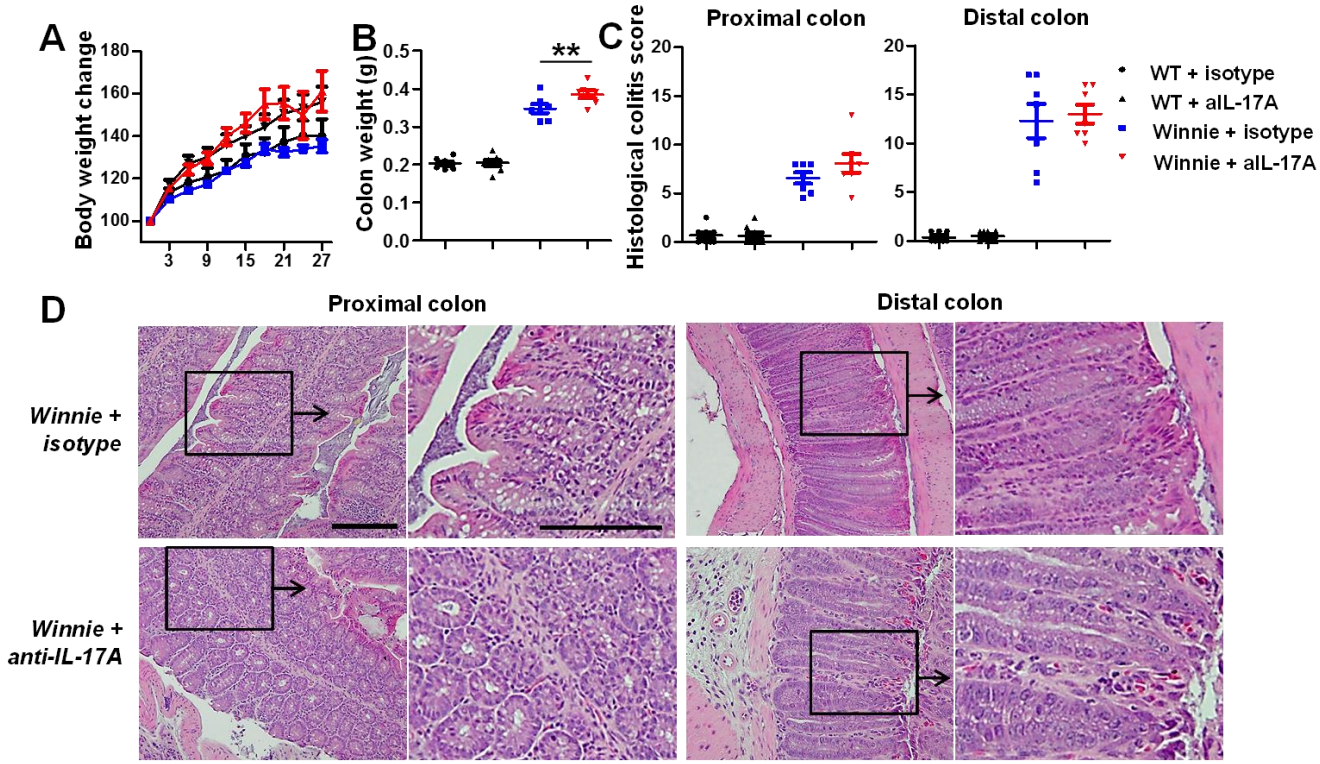


Figure 5.2 Neutralizing IL-17A fails to ameliorate emerging colitis in *Winnie* mice. *WT* and *Winnie* mice at 4 weeks of age were treated for 4 weeks with anti-IL-17A (aIL-17A) or isotype control antibody and then sacrificed. (A) Body weight. (B) Colon weight. (C) Histological colitis scores. (D) Representative H&E stained proximal and distal colon sections with a colitis score close to the median of the group. Statistics: A, B and C: Mean ± SEM and individual data points, n=7-10, single experiment; One-way ANOVA with Bonferroni's multiple comparison test (P< 0.01). Scale bars = 100 µm.**

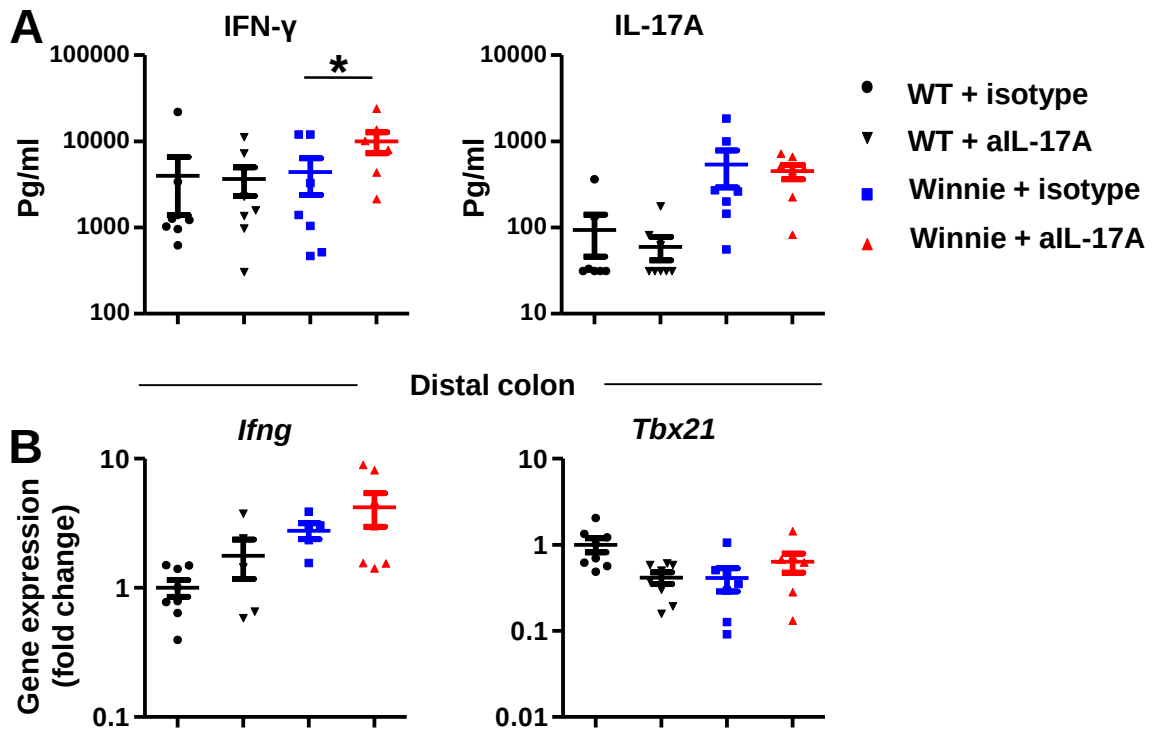


Figure 5.3 Proinflammatory cytokine production in *WT* and *Winnie* mice at 8 weeks of age after 4 weeks treatment with aIL-17A or isotype control antibody. (A) IFN-γ and IL-17A cytokine concentration in isolated mLN leucocyte culture supernatants measured by ELISA. (B) *Ifng* and *Il17a* mRNA expression levels in distal colon of *WT* and *Winnie* mice. Statistics: Mean ± SEM and individual data points, n=7-10, single experiment; One-way ANOVA with Bonferroni's multiple comparison test (*P< 0.05).

5.3.2 Antibody neutralization of IL-17A fails to alleviate established colitis in Winnie mice

Efficacy of the anti-IL-17A antibody was also examined in established colitis using 12 week old *Winnie* mice. After treatment with aIL-17A monoclonal antibody for two weeks, the colon weight of *Winnie* mice did not differ from mice treated with the isotype control (Figure 5.4 A). However, *Winnie* mice receiving aIL-17A antibody had decreased histological colitis scores in the proximal colon (Figure 5.4 B) with less inflammatory cell infiltration (Figure 5.4 B, lower panel). The overall severity of histological colitis did not diminish in the distal colon where colitis is greatest (Figure 5.4 B). mRNA levels of proinflammatory cytokine genes, *Ifnγ, Il17a* and Il1b, did not change in either the proximal or distal colon (Figure 5.5) suggesting that blocking IL-17A alone is not sufficient to suppress established colitis in *Winnie* mice.

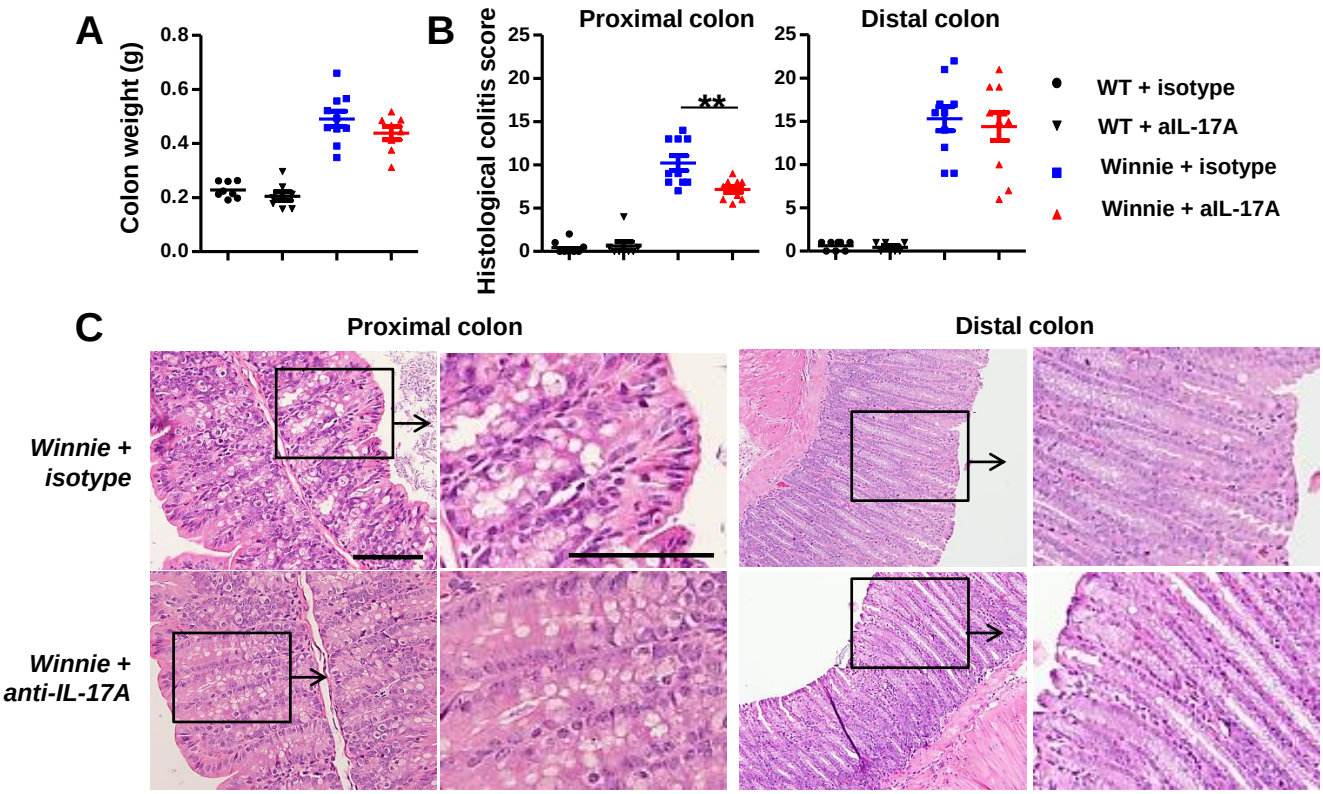


Figure 5.4 Neutralizing IL-17A fails to ameliorate established colitis in *Winnie* mice. C57BL/6 (WT) and *Winnie* mice at 12 weeks of age were treated for 2 weeks with anti-IL-17A (aIL-17A) or isotype control antibodies and then sacrificed. (A) Colon weight. (B) Histological colitis scores. (C) Representative H&E stained proximal and distal colon sections with a colitis score close to the median of the group. Statistics: A and B: Mean ± SEM and individual data points, n=7-10, single experiment; One-way ANOVA with Bonferroni's multiple comparison test (P< 0.01). Scale bars = 100 µm.**

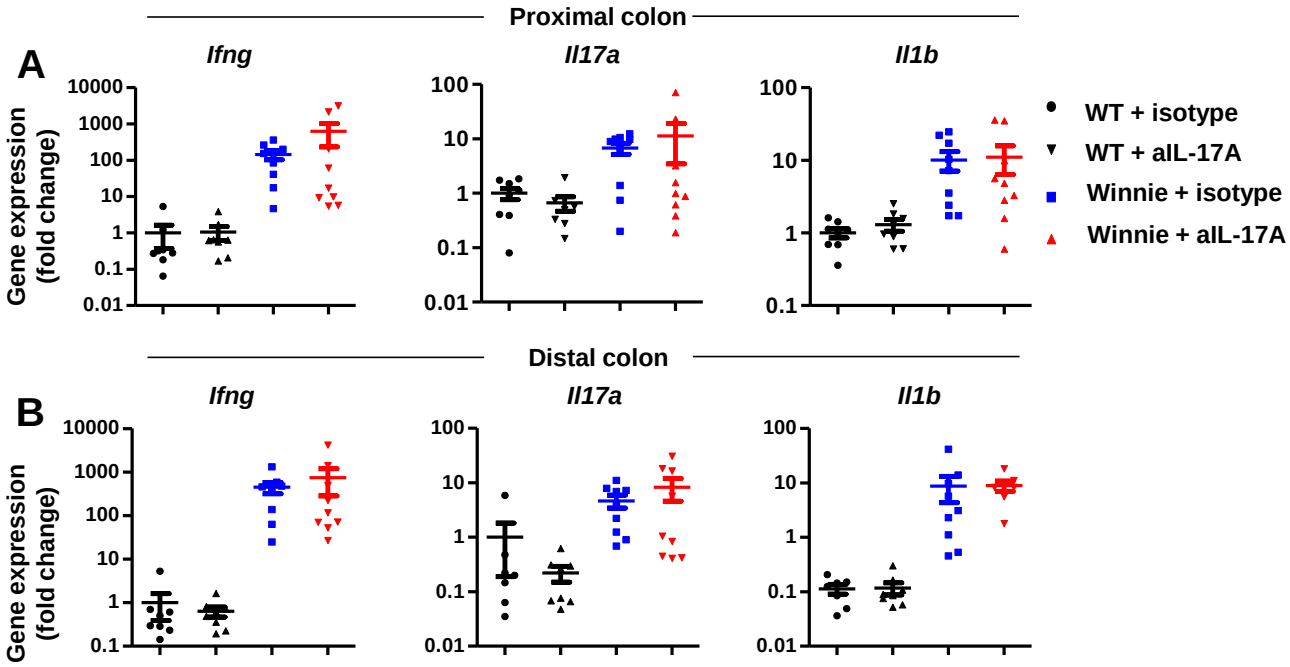


Figure 5.5 mRNA expression of proinflammatory cytokine genes in WT and *Winnie* mice at 12 weeks of age treated for 2 weeks with anti-IL-17A (aIL-17A) or isotype control antibodies and then sacrificed. (A) Proximal colon and (B) distal colon cytokine gene expression. Statistics: Mean ± SEM and individual data points, n=7-10, single experiment; One-way ANOVA with Bonferroni's multiple comparison test.

5.3.3 Antibody neutralization of IL-17Ra does not alleviate established colitis in Winnie mice

To further confirm the role of IL-17A signaling in intestinal colitis, 12 week old *Winnie* mice with established colitis were treated with anti-IL-17Ra monoclonal antibody (aIL-17Ra) for 2 weeks. Similarly, two weeks treatment of 12 week old *Winnie* mice with aIL-17Ra did not reduce intestinal inflammation, with colon weight (Figure 5.6 A) and histological colitis scores (Figure 5.6 B) unchanged compared to *Winnie* receiving isotype control antibody. In fact, increased mucosal neutrophil recruitment was observed in the distal colon of *Winnie* mice treated with anti-IL-17Ra antibody (Figure 5.6 B lower panel), suggesting that the IL-17Ra mediated signaling is not the dominant driver of neutrophil recruitment in the intestinal mucosa. IFN-γ and IL-17A cytokine production by mLN leucocytes did not differ after the treatment. Overall, in contrast to genetic deficiency, blocking the IL-17A-IL-17Ra axis with neutralizing antibodies was insufficient to ameliorate *Winnie* colitis.

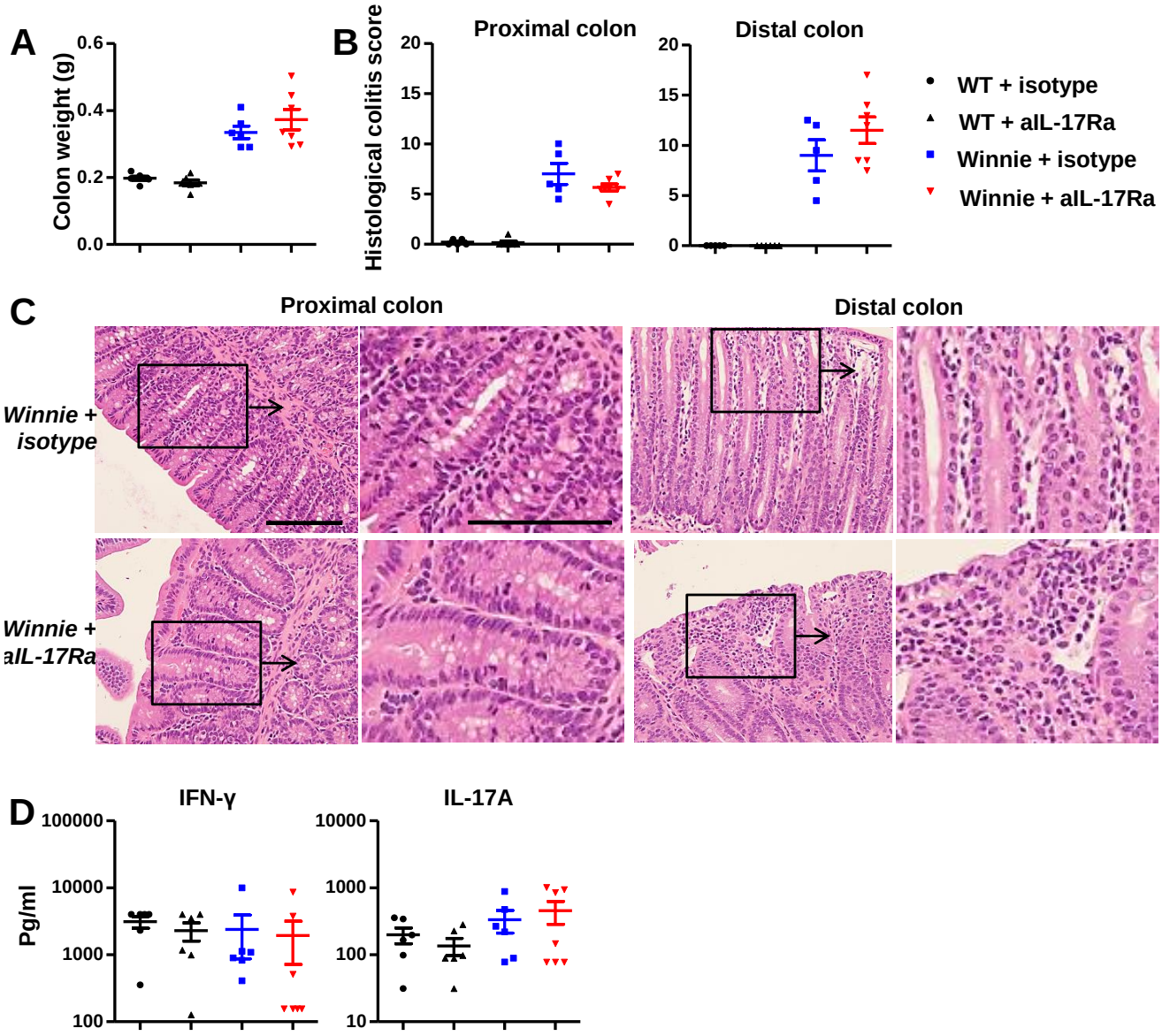


Figure 5.6 Neutralizing IL-17Ra fails to ameliorate established colitis in *Winnie* mice. WT and *Winnie* mice at 12 weeks of age were treated for 2 weeks with anti-IL-17Ra (aIL-17Ra) or isotype control antibody. (A) Colon weight. (B) Histological colitis scores and (C) Representative H&E stained sections of the proximal and distal colon with a colitis score close to the median of the group. (D) IFN-γ and IL-17A cytokine concentrations in isolated mLN leucocyte culture supernatants measured by ELISA. Statistics: A, B and C: Mean ± SEM and individual data points, n=7-10, single experiment; One-way ANOVA with Bonferroni's multiple comparison test (P< 0.01). Scale bars = 100 µm.**

5.3.4 Antibody treatments against other T_H17 associated cytokines fails to show efficacy in reducing colitis severity

As the T_H17 response has a strong proinflammatory phenotype, effector cytokines of T_H17 cells became promising candidates to target in suppressing intestinal inflammation. In a pilot experiment, the efficacy of antibodies against T_H17-associated cytokines other than IL-17A was examined in *Winnie* mice with established colitis. Unfortunately, anti-IL-17F alone, anti-IL-17E alone and anti-IL-17F in combination with anti-IL-17A treatment did not ameliorate *Winnie* colitis. Indeed, 2 weeks anti-IL-17F treatment increased colon weight and inflammatory cell infiltration, indicating more severe intestinal inflammation developed compared to the isotype control group (Figure 5.7). In summary, this small underpowered study provided no evidence that targeting T_H17 cytokines other than IL-17A, or combinations of these cytokines, by monoclonal antibodies is likely to be effective in suppressing colitis in the *Winnie* mice.

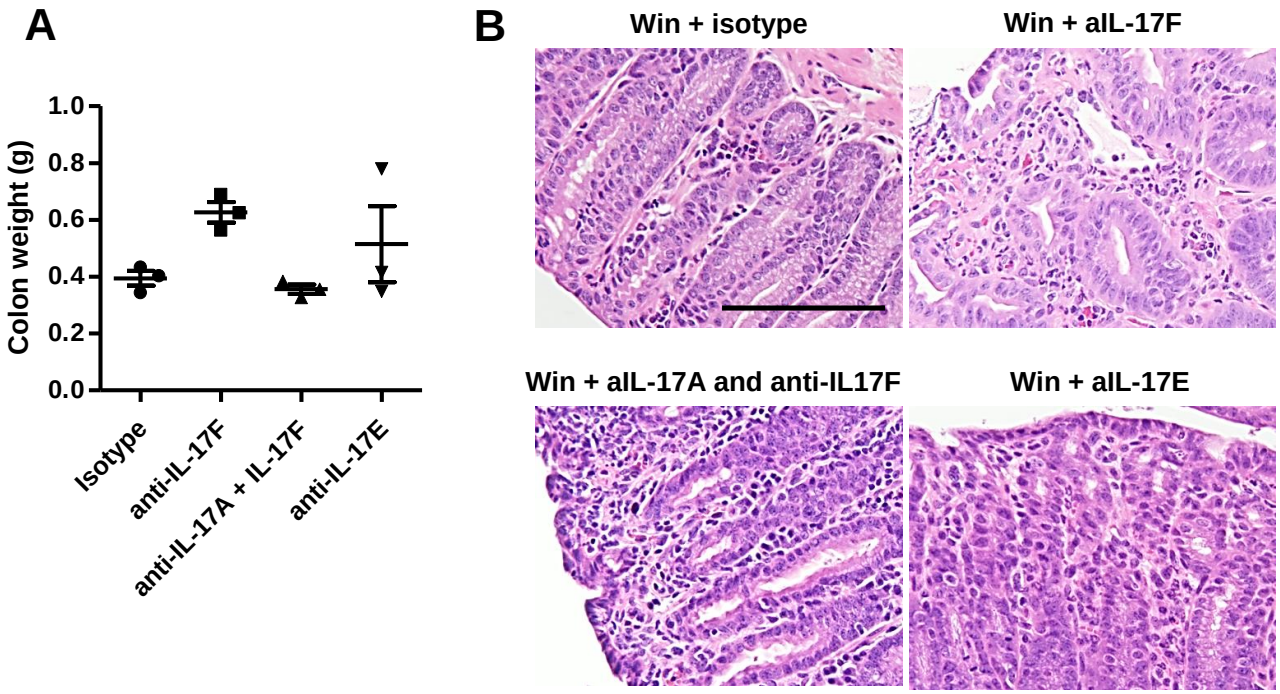


Figure 5.7 12 week old *Winnie* mice treated with anti-IL-17F, anti-IL-17E, IL-17F in combination with anti-IL-17A, or isotype control antibody for 2 weeks. (A) Colon weight of mice on the day of sacrifice. (B) Representative H&E stained sections of the distal colon with a colitis score close to the median of the group. Statistics: mean and individual data points; n=3, mixed gender, single experiment. Scale bars = 100 µm.

5.4. Discussion

In this chapter, the efficacy of blocking T_H17 associated cytokines or the cytokine receptor using monoclonal antibodies has been assessed. Results have shown that neutralizing either T_H17 effector cytokines or the IL-17Ra cytokine receptor does not suppress colitis in the *Winnie* model. Although IL-17A is not responsible for the induction of experimental colitis, it exerts a strong proinflammatory function on myeloid and mesenchymal cells to induce granulocyte colony-stimulating factor and IL-6 production, which subsequently promotes recruitment of neutrophils and monocytes (Xu and Cao, 2010). This function of IL-17A links adaptive and innate immunity with implications for both host defence and tissue inflammation. A recent study demonstrates that the presence of T_H17 cells requires segmented filamentous bacteria colonization in mice (Yang et al., 2014), suggesting that the commensal microbiota could be involved in the evolution of tissue inflammation processes. IL-17A also stimulates antimicrobial molecule production and enhances host defense against extra cellular pathogens at various mucosal sites (Khader et al., 2009), potentially providing an explanation for the results of a clinical trial in which treating active Crohn's disease patients using anti-IL-17A monoclonal antibody not only failed to show efficacy, but increased the incidence of fungal infection (Hueber et al., 2012). Our finding that neutralizing IL-17A failed to suppress intestinal inflammation is consistent with the results of this clinical trial. Bacterial or fungal infections were not observed in our experiments; however, evidence of infection was not definitively sought. Evidence from human clinical trials suggest that anti-IL-17A antibody treatment is effective in treating autoimmune disease involving inflammatory T_H17 responses like psoriasis, rheumatoid arthritis, and uveitis (Hueber et al., 2010; Leonardi et al., 2012; Papp et al., 2012). However, the pathogenesis of inflammatory bowel disease is closely entwined with the luminal microbiota, unlike the other autoimmune diseases that involve tissues with less or no microbial exposure. While the physiological role of IL-17A in intestinal immunity is obviously complex, neutralizing IL-17A may perturb fine tuning of the immune balance between host defence and tissue inflammation.

IL-17A signals through a heterodimeric receptor complex including the IL-17Ra and IL-17Rc subunits (Toy et al., 2006). IL-17Ra is ubiquitously expressed on various cell types and involved in IL-17A and IL-17F signaling (Korn et al., 2009). IL-17Ra is critical for inducing neutrophil migration during acute lung infection (Crowe et al., 2009), and is indispensable for IL-17A-mediated IL-6 and chemokine production in rheumatoid arthritis

(Zrioual et al., 2008). IL-17Ra is able to activate NF-κB and the Janus kinase 1-associated phosphoinositide 3-kinase signaling pathway independently (Huang et al., 2007; Yao et al., 1995), suggesting its involvement in infection and inflammation. However, our results show that blocking IL-17Ra is insufficient to suppress colitis and limit tissue neutrophil recruitment, and that targeting IL-17Ra is unlikely to be effective in treating intestinal inflammation.

As discussed in the previous chapter, IL-17A and IL-17F share the most homology within the IL-17 effector cytokine family. Both cytokines have proinflammatory functions, with IL-17F less potent than IL-17A in recruiting neutrophils and stimulating downstream inflammatory cytokine production (Leppkes et al., 2009; Yang et al., 2008b). Surprisingly, results from this chapter show that neutralizing IL-17A and IL-17F concomitantly with monoclonal antibodies does not alter colitis severity in *Winnie* mice, indicating other proinflammatory cytokines are more substantial contributors to intestinal immunopathology.

IL-17E, also known as IL-25, is a T_H17 family cytokine that can also be produced by T_H2 cells, mast cells, eosinophils and basophils (Ikeda et al., 2003; Tamachi et al., 2006) involving in T_H2 cell expansion and allergic reactions (Angkasekwinai et al., 2007; Fort et al., 2001). IL-17E has been shown to inhibit IL-17A and IFN-γ production and suppress T_H17-mediated pathology, suggesting its protective role in antagonizing T_H17 responses (Kleinschek et al., 2007; Owyang et al., 2006). Recombinant IL-17E treatment was reported to ameliorate chemically-induced colitis and decrease IL-12 and IL-23 levels in the intestinal mucosa (Caruso et al., 2009), indicating that IL-17E is important in maintaining intestinal homeostasis. Therefore, it is not surprising to observe that anti-IL-17E treatment increased the colon weight of *Winnie* mice. However, a properly powered experiment with sufficient numbers of mice needs to be performed before we can confidently conclude that neutralization of IL-17F and IL-17E is not therapeutic in *Winnie* mice.

In summary, neutralizing T_H17-associated effector cytokines or cytokine receptor fails to alleviate the intestinal inflammation in *Winnie* mice. This could be due to the dual function of T_H17 cells in fighting against pathogens and inducing tissue inflammation. As targeting T_H17-associated effector cytokines or cytokine receptor were not able to

suppress *Winnie* colitis, antibodies against the critical upstream cytokine of the T_H17 pathway, IL-23, were tested for their in suppressing *Winnie* colitis in the next chapter.

6.0 Antibody Neutralization of IL-23 Suppresses *Winnie* Colitis

6.1. Overview

Given that blocking IL-17A, the major effector cytokine of proinflammatory T_H17 cells, did not ameliorate colitis in *Winnie* mice, we hypothesized that blocking IL-23, the upstream cytokine of IL-17A producing immune cells, would reduce intestinal inflammation.

IL-23, comprising p40 (shared with IL-12) and p19 subunits, is a member of the IL-12 cytokine family known to promote and sustain proinflammatory T_H17 T cells and is linked by genetic and experimental evidence to the pathogenesis of IBD. IL-23 is secreted by antigen presenting cells upon exposure to pathogens (Langrish et al., 2004). Lamina propria $CD103^+CD11b^+$ dendritic cells are able to produce IL-23 in response to the bacterial TLR5-ligand, flagellin (Kinnebrew et al., 2012). In response to IL-23, effector/memory T cells actively produce proinflammatory cytokines, including IL-17A, thereby driving local tissue inflammation (Aggarwal et al., 2003). In a *Helicobacter hepaticus* infection and T cell transfer induced colitis model, *p19*$^{-/-}$*Rag1*$^{-/-}$ recipients lacking the ability to produce IL-23 had attenuated transfer colitis compared to IL-23-producing *Rag1*$^{-/-}$ recipients (Kullberg et al., 2006). In addition to the direct effect on effector T cells, IL-23-dependent ILCs producing IL-17A and IL-17F have been found in Crohn's disease patients (Geremia et al., 2011). These observations are supported by experimental studies in mice lacking T cells, showing that IL-23 can drive innate intestinal pathology through IL-17A production by ILCs (Buonocore et al., 2010a). IL-23 also suppresses Treg differentiation, suggesting that in the presence of IL-23 intestinal inflammation could develop due to reduced Treg-mediated immunosuppression (Izcue et al., 2008). In addition, human GWAS demonstrate that an *IL23R* gene polymorphism resulting in impaired IL-23 receptor signaling confers strong protection against Crohn's disease and ulcerative colitis (Duerr et al., 2006a). Thus human genetics indicates that functional IL-23 signalling is required for the development of intestinal inflammation. Due to IL-23's multiple mechanisms of actions on a range of effector and immunomodulatory immune cells, targeting IL-23 to suppress intestinal inflammation has promise as an approach to treat IBD.

ABT-874 and Ustekinumab (CNTO 1275), two humanized monoclonal antibodies against the p40 subunit of IL-12/23 cytokines, have been trialed in treating moderate-to-severe Crohn's disease. Crohn's disease patients receiving ABT-874 antibody demonstrated an increased clinical response rate compared to a placebo group (Mannon et al., 2004). Ustekinumab was effective in moderate-to-severe Crohn's disease patients in a double-blinded cross-over trial (Sandborn et al., 2008). Ustekinumab also significantly increased response and remission rates as a maintenance therapy in treating Crohn's disease patients refractory to anti-TNF therapy (Sandborn et al., 2012). BI 655066, an antibody against IL-23 only, is being tested in an ongoing double-blind, placebo-controlled, phase II dose-ranging study in patients with moderate-to-severe Crohn's disease (NCT02031276, ClinicalTrials.gov). However, these antibodies are yet to be systematically tested in ulcerative colitis.

Winnie spontaneous colitis arises from an epithelial defect due to a single missense mutation in the *Muc2* mucin gene (Heazlewood et al., 2008) (see 1.4.4 for detail). The unfolded or misfolded MUC2 accumulates in the ER of the intestinal secretory goblet cells and causes ER stress. ER stress refers to a physiological state that unfolded or misfolded proteins accumulate in the ER lumen, due to increased demand or improper protein folding which further cause imbalances between the protein folding load and the capacity of the ER (Zhang and Kaufman, 2008). To prevent the accumulation of unfolded or misfolded proteins in the ER, eukaryotic cells have evolved a mechanism to cope with ER stress and to resolve the protein folding defect – a response referred to as the unfolded protein response (UPR) (Schroder and Kaufman, 2005). The main UPR signaling cascade is initiated by three major ER localized proteins. They are inositol-requiring 1α (IRE1α), double-stranded RNA-dependent protein kinase (PKR)-like ER kinase (PERK) and activating transcription factor 6 (ATF6) (Ron and Walter, 2007; Schroder and Kaufman, 2005). Severe or unresolved ER stress triggers downstream inflammatory responses through multiple UPR-dependent and independent mechanisms (Hasnain et al., 2012; McGuckin et al., 2010). Intestinal goblet cell ER stress can lead to depletion of the mucus barrier and increased microbial exposure (Eri et al., 2011).

ER stress and the UPR are closely interconnected with inflammation through various mechanisms. Although the UPR is effective in maintaining ER homeostasis, prolonged ER stress can trigger inflammatory signaling (Hasnain et al., 2012) and even

secretory cell apoptosis (Ron and Walter, 2007; Schroder and Kaufman, 2005). As major mediators of inflammation, it is emerging that inflammatory cytokines also play a vital role in regulating ER stress in secretory cells. Besides the fact that intracellular stress signals can activate the NF-kB pathway resulting in inflammatory cytokine production, some cytokines can also promote ER stress by increasing oxidative stress, and thereby exacerbate inflammation. It has been reported that TNF-α induces ER stress by modifying oxidative stress (Xue et al., 2005), while other cytokines like IL-10 and IL-22 alleviate oxidative stress (Shkoda et al., 2007). A recently published finding from our group demonstrated that IL-23 is a very potent cytokine in inducing ER stress and oxidative stress in pancreatic β-cells (Hasnain SZ, 2014), which have many similarities to goblet cells, suggesting a possible role for IL-23 in directly driving pathology in intestinal secretory cells thereby exacerbating pathology.

In order to define the capacity of anti-IL-23 to suppress intestinal inflammation and further study IL-23's mechanism of actions, particularly with respect to effects on goblet cell ER stress, I tested the efficacy of monoclonal antibodies against the p40 or p19 subunits of IL-23 in *Winnie* mice with emerging and established colitis. Histological colitis, cytokine gene expression, inflammatory cell infiltration and goblet cell phenotype were assessed to determine the relative efficacy of anti-p40 and anti-p19 antibodies. *In vitro* assays were performed to further investigate the effect of IL-23 on colonic epithelial cells in the context of inducing ER stress and oxidative stress.

6.2. Methods

Antibody neutralization of IL-23 was carried out in three colitis models: (a) 5 to 6 week old *Winnie* mice with emerging colitis were treated with anti-p19 (ap19) and anti-p40 (ap40) antibodies for 2 weeks; (b) 5 to 6 week old *Winnie* mice were treated with anti-p19 (ap19) and isotype antibodies for 4 weeks, and (c) 12 week old *Winnie* mice with established colitis were treated with anti-p19 and anti-p40 antibodies for 2 weeks. At the end of the experiment, mice were sacrificed to examine the intestinal inflammation. On the day of sacrifice, intestinal tissue was collected to assess the colitis severity (see 3.12 for details). The whole colon, caecum and terminal ilium were dissected and colon length was measured. Colon weight was assessed after removal luminal contents (see 3.10 for details). Proximal and distal colons were dissected for histological colitis examination (see

3.19 for details). Total tissue RNA was isolated and reverse transcribed to cDNA (see 3.15 and 3.16 for details). Proinflammatory cytokine gene expression was measured in both proximal and distal colon tissue samples. Expression of each gene was corrected to each sample's housekeeping gene before being normalized to the average of the relevant control group (see 3.17.2 for details). H&E staining of intestinal sections was carried out to assess histological pathology. Histological colitis was quantified using the chronic or acute colitis scoring system (see 3.19 for details). Representative sections of proximal and distal colon with a colitis score close to the median of the group were presented.

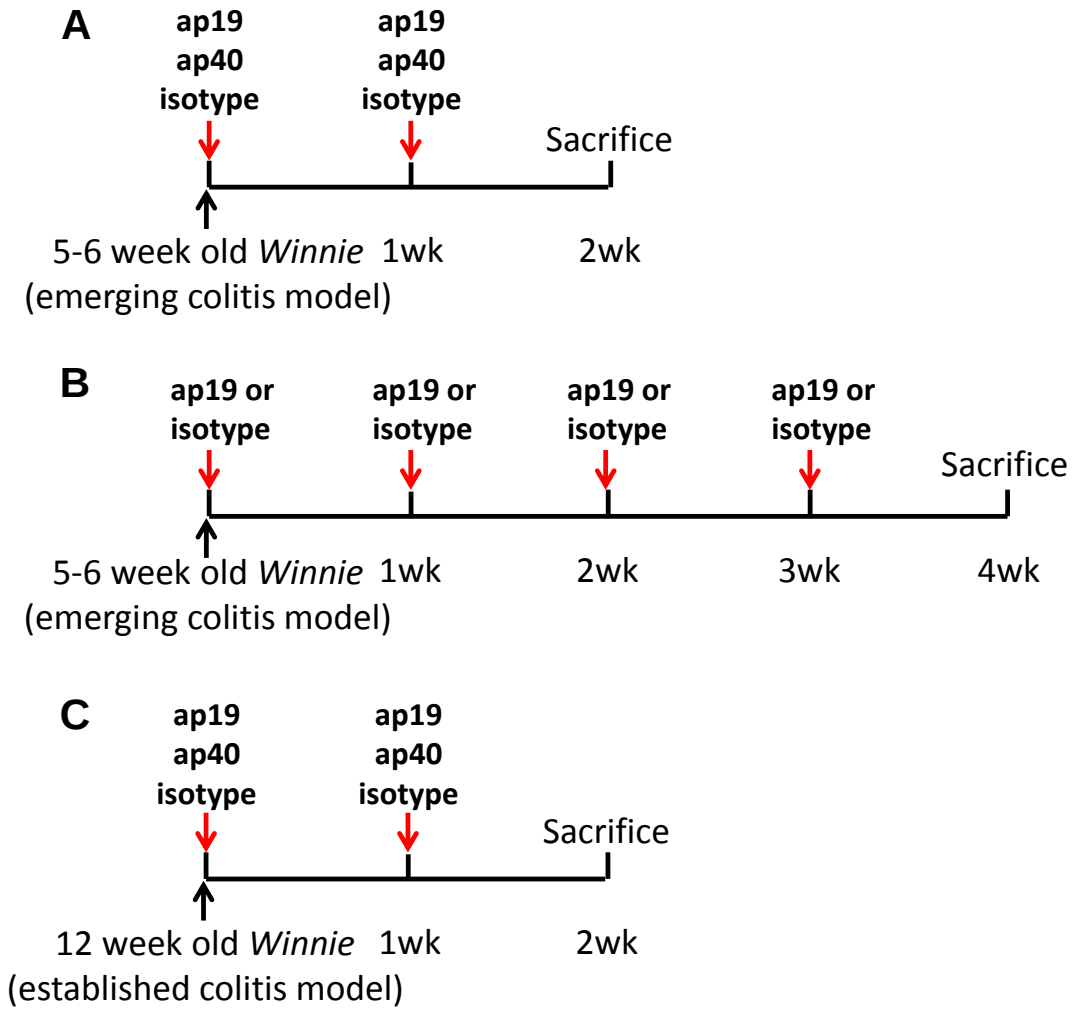


Figure 6.1 Antibody treatment regimens for C57BL/6 (WT) and *Winnie* mice at different ages. (A) 5 to 6 week old *Winnie* mice with emerging colitis were treated with anti-p19 (ap19) and anti-p40 (ap40) antibodies for 2 weeks. (B) 5 to 6 week old *Winnie* mice with emerging colitis were treated with anti-p19 antibody for 4 weeks. (C) 12 week old *Winnie* mice with established colitis were treated with anti-p19 and anti-p40 antibodies for 2 weeks.

Immunohistochemistry for Ly6G/6C was performed to assess neutrophil infiltration in the colon (see 3.18.2 for details). Morphometry was used to quantify staining as described in 3.18.5. To investigate whether IL-23 is involved in neutrophil activation, multiple assays were carried out. Firstly, IL-23R expression on freshly isolated mouse peritoneal neutrophils was determined by flow cytometry. Isolated mouse peritoneal neutrophils were co-cultured with or without 50 ng/ml IL-23 for 2 h, intra-cellular ROS levels were measured by the DCFDA assay (see 3.4 for details) after treatment to compare the activation state of neutrophils. To examine if IL-23 is involved in neutrophil recruitment *in vivo*, 12 week old C57BL/6 mice were administered IL-23 (50 µg/kg body weight, i.p.) 2 h before being sacrificed. ROS and oxidative stress levels were assessed by the DCFDA assay (see 3.4 for details) and the DHE dye oxidation assay (see 3.5 for details) in isolated peritoneal neutrophils with or without IL-23 pre-exposure.

Alcian blue and PAS staining was used to examine mucin production by goblet cells (see 3.18.4 for details). Morphometry was used to quantify staining as described in 3.18.5. To confirm the *in vivo* findings, the human colonic epithelial cell line that contains MUC2-producing goblet cells, LS174T, was treated with IL-23 (50 ng/ml). Various treatment regimens were used and intra-cellular ROS levels, ER stress, chemokine IL-8 secretion and MUC2 production were measured to study the effects of IL-23 on colonic epithelial cells.

6.3. Results

6.3.1 Neutralizing IL-23 alleviates emerging colitis in Winnie mice

5 to 6 week old *Winnie* mice with emerging colitis were treated with monoclonal antibodies against the p40 or p19 subunit of IL-23 for two weeks. Although anti-p19 antibody treatment did not modify the colon weight (Figure 6.2 A), the histological colitis score was reduced in both the proximal and distal colon of *Winnie* mice (Figure 6.2 B). H&E staining of colon sections revealed that *Winnie* mice receiving anti-p19 antibody had less inflammatory cell infiltration and improved crypt structure compared to untreated mice (Figure 6.2 C). Inflammatory cytokine gene expression was not elevated in the proximal colon of young *Winnie* mice, and was not modified by the anti-p19 antibody treatment (Figure 6.2 D). However, cytokine and chemokine genes were increased in the distal colon of *Winnie* mice and anti-p19 treatment significantly downregulated *Il17a, Il1b* and *Mip2α* mRNA expression (Figure 6.2 E), suggesting that IL-23-driven production of the

proinflammatory cytokines, IL-17A, IL-1β and chemokine Mip-2α, by inflammatory cells contributes to the intestinal pathology in *Winnie* emerging colitis. Anti-p40 antibody treatment also downregulated *Il17a, Il1b* and *Mip2α* mRNA expression in the distal mucosa of *Winnie* mice (Figure 6.2 E), but it did not alleviate the overall histological colitis. The anti-p40 antibody, which neutralizes both IL-12 and IL-23, was not as effective as anti-p19 antibody in ameliorating *Winnie* colitis. Of potential relevance, though not significant, anti-p40 appeared to reduce expression of the T_H1 cytokine, *Ifng*, whereas anti-p19 did not, potentially explaining the differing effects of targeting IL-23 alone vs IL-12 and IL-23.

6.3.2 Neutralizing IL-23 suppresses progression of colitis in Winnie mice

Winnie spontaneous colitis is progressive with the most severe inflammation in the distal colon (Eri et al., 2011). Based on the results from the short term experiment in 6.3.1, in an attempt to mimic maintenance therapy in the clinic, 5 to 6 week old *Winnie* mice were treated with anti-p19 antibody for four weeks to assess its efficacy in alleviating colitis progression. After four weeks treatment, *Winnie* mice receiving anti-p19 antibody treatments had lower histological colitis scores in the distal colon (Figure 6.3 B), although the overall colon weight (Figure 6.3 A) and histological colitis score in the proximal colon (Figure 6.3 B) were similar to isotype treated mice. Anti-p19 antibody treated *Winnie* mice also exhibited improved crypt structure and reduced inflammatory cell infiltration compared to untreated mice (Figure 6.3 C). Inflammatory cytokine and chemokine genes were elevated in both proximal and distal colon of *Winnie* mice and anti-p19 treatment significantly downregulated *Il17a, Il1b* and *Mip2α* mRNA expression (Figure 6.2 D and E), suggesting that anti-IL-23 antibody alleviated the progression of colitis characteristic of *Winnie* mice by reducing production of proinflammatory cytokines and chemokines in the intestinal mucosa. Similar to the previous experiment, *Ifng* gene expression in the proximal and distal colon did not change with anti-p19 treatment, indicating that IFN-γ may not contribute to the overall immunopathology in *Winnie* colitis as it becomes more severe with age.

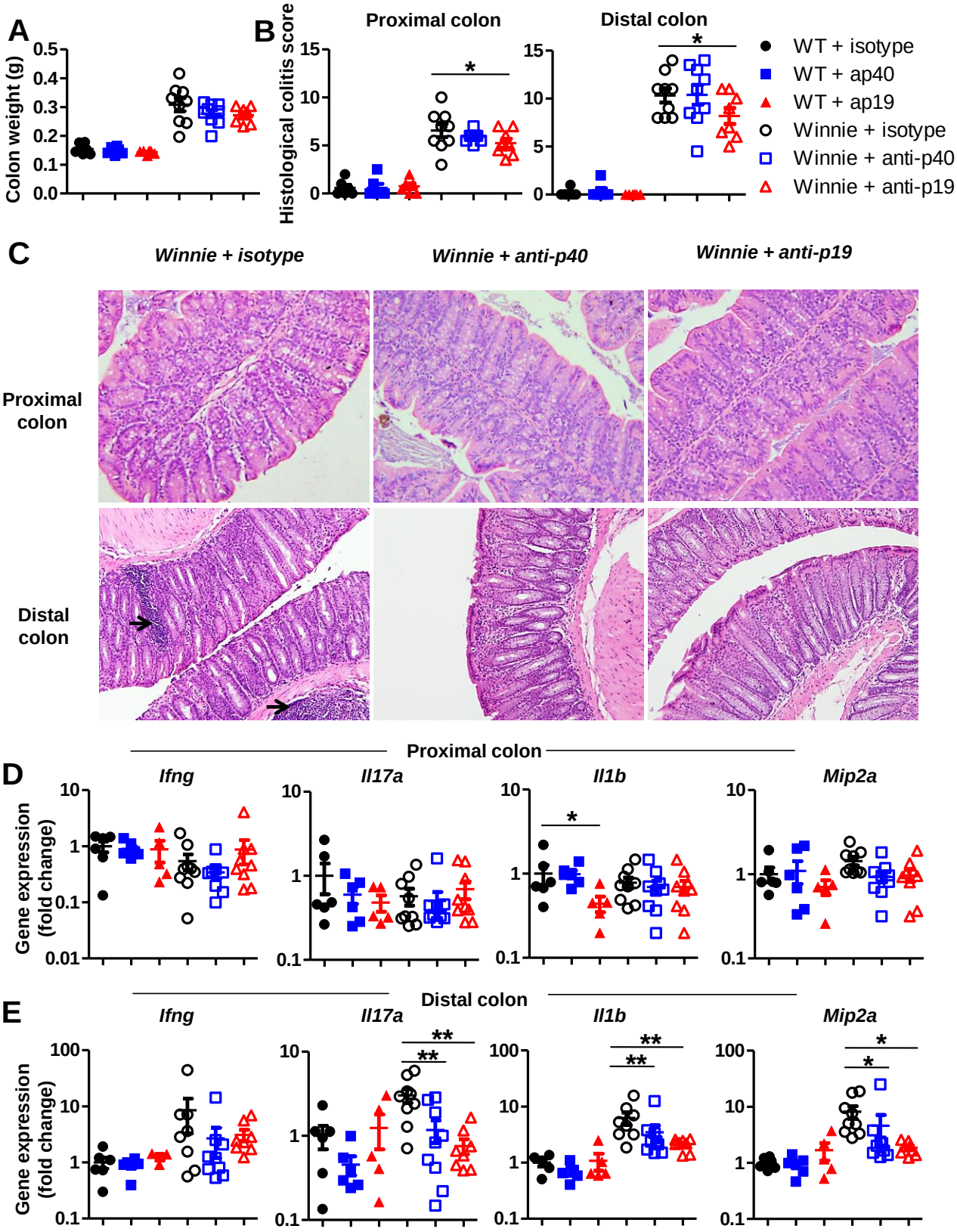


Figure 6.2 Neutralizing IL-23 using anti-p19 antibody ameliorates emerging colitis in *Winnie* mice. WT and *Winnie* mice at 5-6 weeks of age were treated with anti-p40, anti-p19 or isotype antibodies for 2 weeks. (A) Colon weight. (B) Histological colitis scores. (C) Representative H&E

stained sections of proximal and distal colon with a colitis score close to the median of the group (black arrows indicate increased inflammatory cell infiltration). (D) Proximal colon and (E) distal colon cytokine and chemokine gene expression relative to the mean of untreated C57BL/6 mice. Statistics: Mean ± SEM and individual data points, n=6-10, mixed gender, single experiment; B, D and E: One-way ANOVA with Bonferroni's multiple comparison test (*P< 0.05, **P< 0.01). Scale bars = 100 µm.

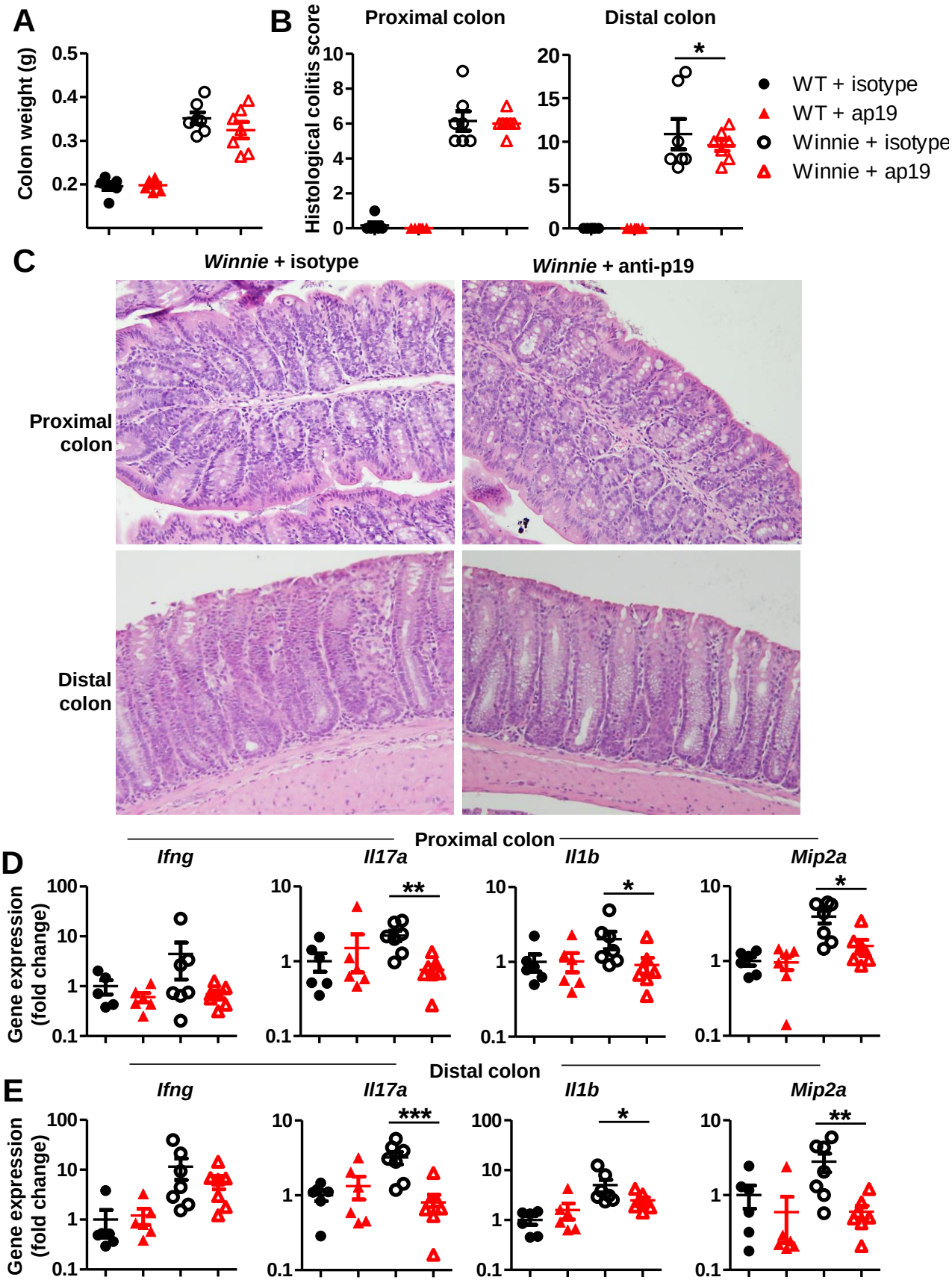


Figure 6.3 Neutralizing IL-23 using anti-p19 antibody ameliorates emerging colitis in *Winnie* mice. WT and *Winnie* mice at 5 weeks of age were treated with anti-p19 or isotype antibodies

for 4 weeks. (A) Colon weight. (B) Histological colitis scores. (C) Representative H&E stained sections of proximal and distal colon with a colitis score close to the median of the group. (D) Proximal colon and (E) distal colon cytokine and chemokine gene expression relative to the mean of untreated C57BL/6 mice. Statistics: Mean ± SEM and individual data points, n=6-7, mixed gender, single experiment; B, D and E: One-way ANOVA with Bonferroni's multiple comparison test (*P< 0.05, **P< 0.01, ***P<0.001). Scale bars = 100 μm.

6.3.3 Neutralizing IL-23 ameliorates established colitis in Winnie mice

The intestinal inflammation of *Winnie* mice progresses with age accompanied by increasing IL-23 expression (Figure 6.4 A) and prominent distal colitis by 12 weeks of age featuring goblet cell depletion, epithelial damage, disruption of crypt architecture and substantial neutrophilic infiltration (Figure 6.4 B). To examine the efficacy of anti-IL-23 antibodies in treating severe intestinal inflammation, 12 week old *Winnie* mice with established colitis were treated with monoclonal antibodies against the p40 or p19 subunit of IL-23 for two weeks. Anti-p19 antibody treatment ameliorated established colitis. Colon weight was reduced (Figure 6.5 A), accompanied by decreased histological colitis scores in the distal colon (Figure 6.5 B), with reduced crypt hyperplasia, epithelial damage and inflammatory cell infiltration (Figure 6.5 C). In contrast, anti-p40 treatment failed to reduce colon weight, nor did it alleviate histological colitis (Figure 6.5 A, B, C). Decreased *Ifnγ* mRNA expression in the distal colon was found after anti-p40 but not anti-p19 treatment, suggesting that blocking IL-12 and IL-23 by using anti-p40 antibody suppressed production T_H1 cytokines. However, decreased IFN-*γ* production did not result in reduced colitis severity, again suggesting that the T_H1 arm of immunity, though activated, may not be a major contributor to mucosal immunopathology in *Winnie* colitis, and could even be somewhat protective. Surprisingly, although anti-p19 treatment suppressed histological colitis, the mRNA levels of *Il17a and Il-1b* in the distal colon of *Winnie* mice did not differ between treatments (Figure 6.5 D, E). This experiment indicates that the efficacy of anti-IL-23 treatment in *Winnie* established colitis may due to mechanisms other than directly suppressing IL-17A-producing immune cells.

As distal colonic neutrophil infiltration is a key feature of *Winnie* colitis, immunohistochemistry of Ly6G/6C was performed to quantify the colonic neutrophil infiltration. Although the Ly6G/6C antigen can also be present on myeloid-derived suppressor cells, positive cells invariably had a polymorphonuclear phenotype and

therefore analysis of Ly6G/6C staining is likely to quite accurately reflect neutrophil content. Two weeks anti-p19 treatment significantly decreased colonic neutrophil infiltration in *Winnie* mice compared to the isotype treated group and was more effective than anti-p40 antibody (Figure 6.6 A, B). IL-17 is regarded as an important neutrophil chemoattractant during T_H17 immune responses, yet *Il17*a gene expression did not change with any of the treatments, therefore the chemokine Mip-2α (*Cxcl2*) and cytokine IL-1β (*Il1b*) also involved in neutrophil recruitment was measured. Although *Mip2α* (Figure 6.6 C) and *Il1b* (Figure 6.5 C,D) gene expression levels were similar in RNA extracted from whole tissue homogenates of the distal colon, *Mip2α* and *Il1b* expression was significantly downregulated in isolated colonic epithelial cells, with both anti-p40 and anti-p19 treatments (Figure 6.6 D), suggesting that IL-23-driven Mip-2α and *Il1b* production by colonic epithelial cells contributes to *Winnie* established colitis. IL-23 dependent epithelial cell chemokine and cytokine production could be explained by (a) IL-23 acting on non-epithelial mucosal cells to trigger production of factors driving epithelial activation and chemokine production, (b) IL-23 having a direct effect on epithelial cells, and/or (c) IL-23 directly acting as a neutrophil chemoattractant.

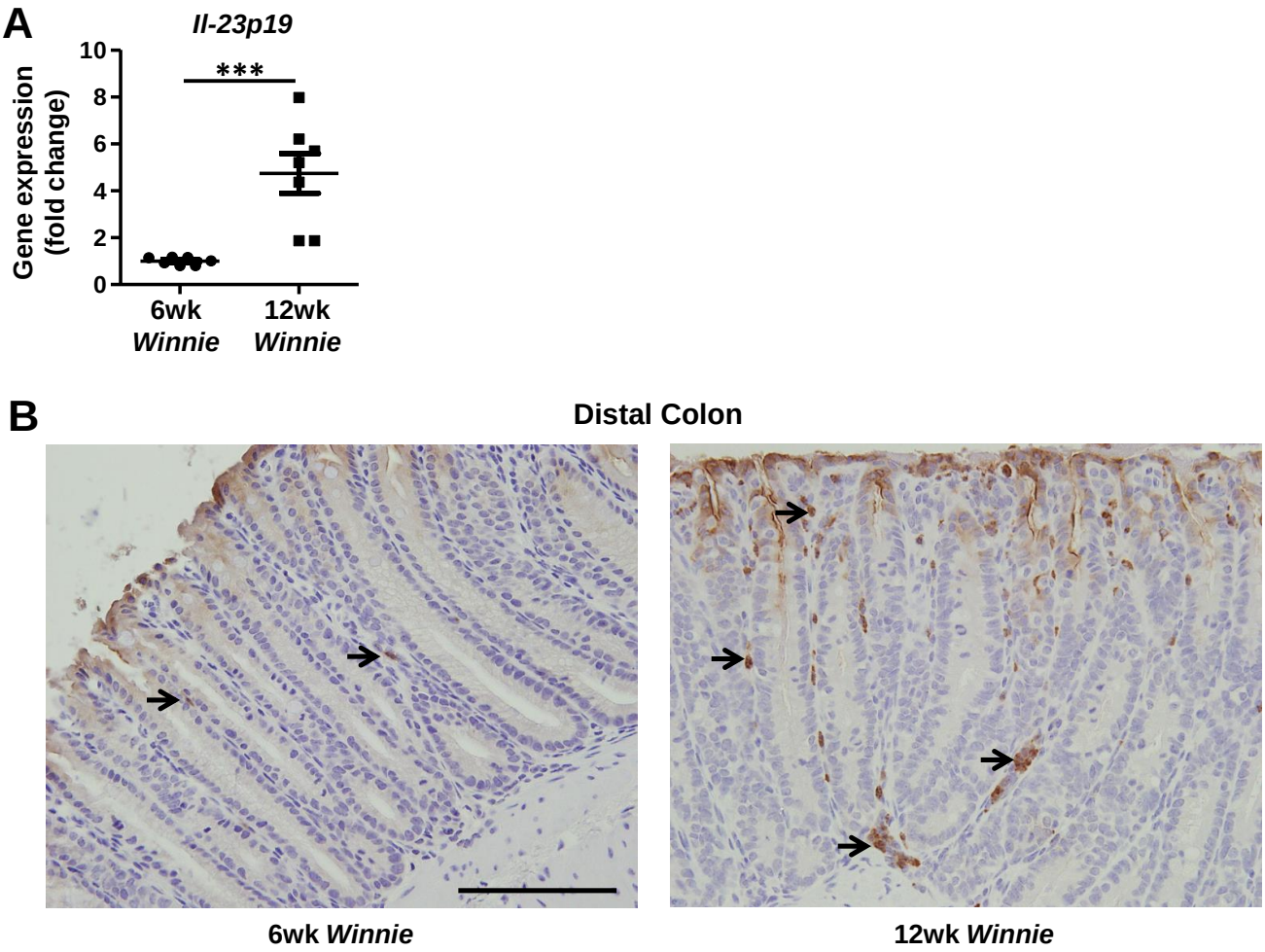


Figure 6.4 12 week old *Winnie* mice have increased *Il23p19* gene expression and distal colonic neutrophil infiltration compared to 6 week old *Winnie* mice. (A) *Il23p19* mRNA expression relative to the mean of the 6 week group. (B) Immunohistochemistry of Ly6G/6C staining (black arrows indicate neutrophils). Statistics: Mean ± SEM and individual data points, n=7, mixed gender, single experiment; A: One-way ANOVA with Bonferroni's multiple comparison test (*P< 0.001). Scale bars = 100 µm.**

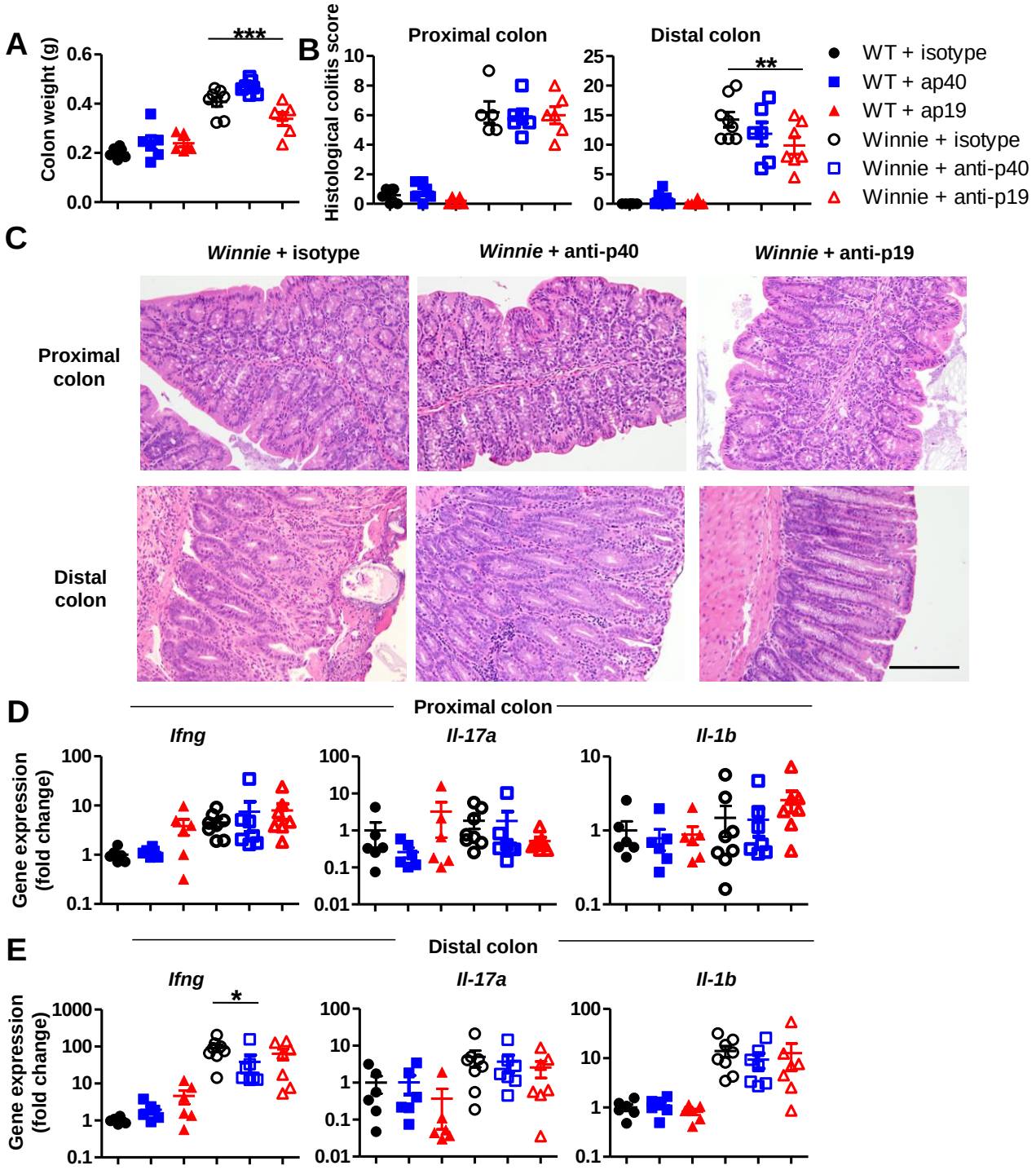


Figure 6.5 Neutralizing IL-23 ameliorates established colitis in *Winnie* mice. *WT* and *Winnie* mice at 12 weeks of age were treated with anti-p40, anti-p19 or isotype antibodies for 2 weeks. (A) Colon weight. (B) Histological colitis scores. (C) Representative H&E stained proximal and distal colon sections with a colitis score close to the median of the group. (D) Proximal colon and (E) distal colon cytokine and chemokine gene expression relative to the mean of untreated C57BL/6 mice. Statistics: Mean ± SEM and individual data points, n=6-8, experiment performed twice,

data from one experiment; A, B, D and E: One-way ANOVA with Bonferroni's multiple comparison test (*P< 0.05, **P< 0.01, ***P< 0.001). Scale bars = 100 µm.

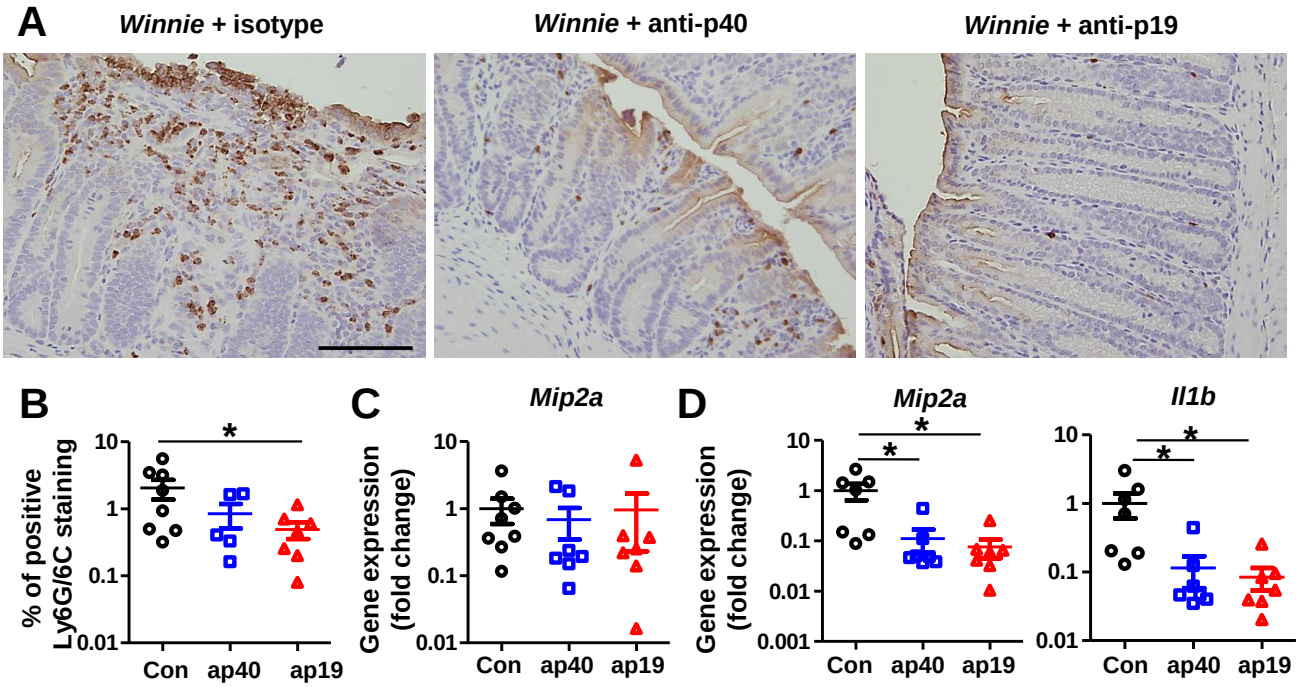


Figure 6.6 *Winnie* mice with established colitis treated for 2 weeks with anti-p19 antibody have reduced neutrophil infiltration in the distal colon. 12 week old *Winnie* mice were treated with isotype, anti-p40 and anti-p19 antibodies for 2 weeks. (A) Representative Ly6G/6C immunohistochemistry showing neutrophil infiltration in distal colon sections. (B) Quantification of Ly6G/6C staining in the distal colon. (C) *Mip2α* gene expression in whole colon tissue. (D) *Mip2α* gene expression in isolated colonic epithelium. Statistics: Mean ± SEM and individual data points, n=5-7, mixed gender, representative data from two experiments; A, B, C and D: One-way ANOVA with Bonferroni's multiple comparison test (*P< 0.05). Scale bars = 100 µm.

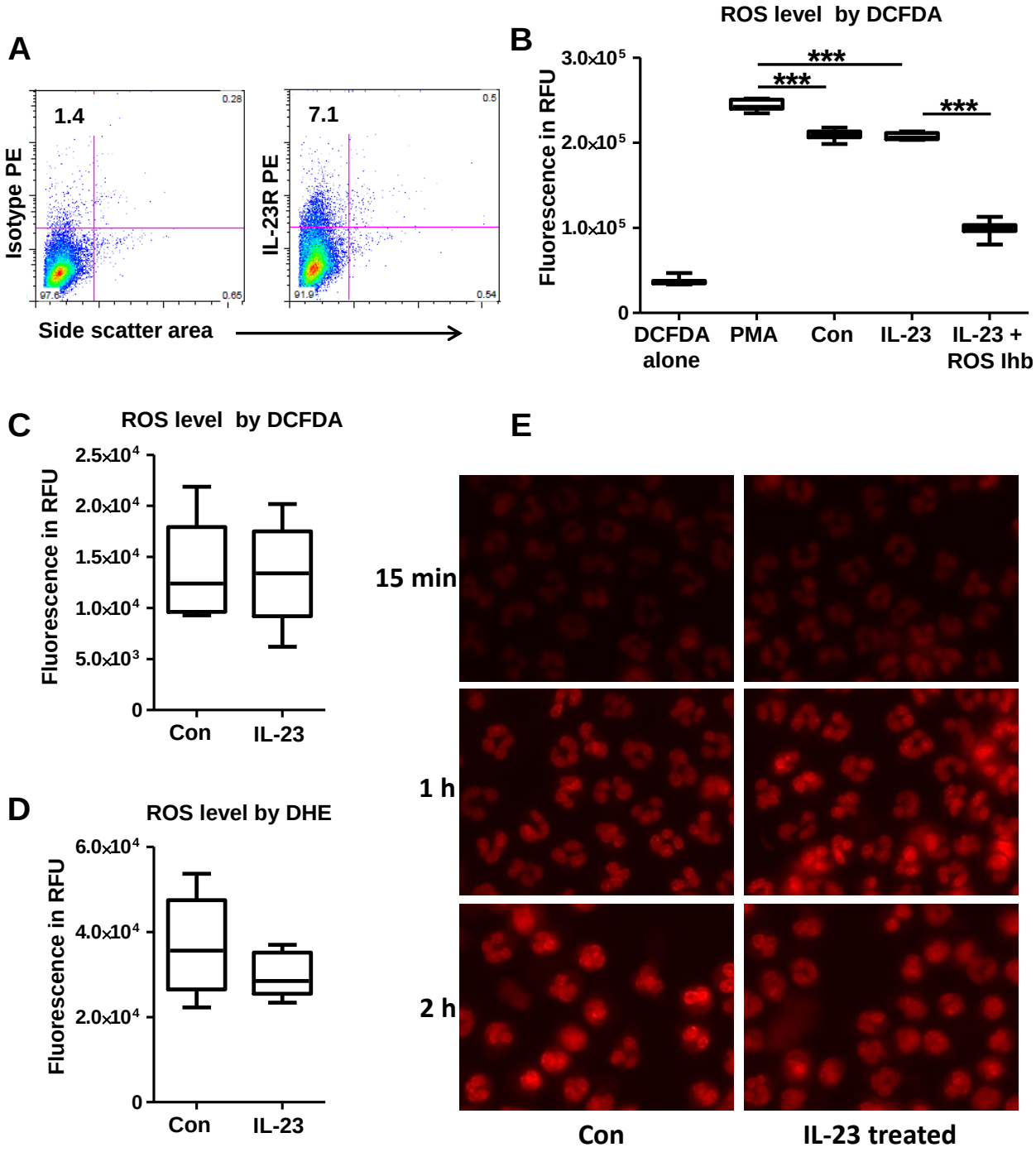


Figure 6.7 IL-23 exposure did not modify the activation state of mouse peritoneal neutrophils. (A) IL-23R expression on isolated mouse peritoneal neutrophils by flow cytometry. (B) Intracellular ROS level in isolated mouse peritoneal neutrophils after treatment with or without 20ng/ml PMA, 50 ng/mL IL-23 or the ROS inhibitor reduced-glutathione (ROS Ihb) for 2 h. (C) Intracellular ROS levels measured by the DCFDA assay in freshly isolated peritoneal neutrophils from naïve C57BL/6 mice or IL-23 (50µg/kg body weight, i.p.) 2 h pre-exposed mice. (D) Intracellular ROS level measured by the DHE assay in mice of (C). (E) DHE dye intensity in neutrophils over 2 h in mice of (C). Statistics: B, C, D: Box plots showing median and inter-

quartile range, n= 5-6, male mice, experiment performed once; B: One-way ANOVA with Bonferroni's multiple comparison test (*P< 0.001).**

Based on the results above, it is possible that IL-23 is directly involved in colonic neutrophil mediated intestinal pathology. A recent report showed that in response to IL-23 human neutrophils increased expression of the IL-17 receptor and IL-17 itself, albeit this study was conducting using very high concentrations of both IL-23 and IL-6 (Taylor et al., 2014). To address this hypothesis, firstly, IL-23R expression was assessed on freshly isolated mouse peritoneal neutrophils by flow cytometry. Around 7% of neutrophils isolated from the peritoneum of casein-stimulated mice were IL-23R positive (Figure 6.7 A). However, *in vitro* IL-23 treatment of isolated neutrophils did not modify the intracellular ROS level as a measure of activation. Notably, control neutrophils have high basal levels of ROS production which can be suppressed by the ROS inhibitor reduced-glutathione, suggesting that neutrophils could be artificially activated during the isolation and purification procedures (Figure 6.7 B). Similar with these finding, peritoneal neutrophils isolated from mice administered IL-23 into the peritoneal cavity had similar levels of intracellular ROS to neutrophils isolated from control C57BL/6 mice (Figure 6.7 C, D). As an indication of whether IL-23 is directly or indirectly a chemoattractant, numbers of neutrophils in the peritoneum were counted, however, no difference was observed. DHE oxidation indicated that neutrophils actively generate ROS over time, but the ROS producing ability was similar between naïve and IL-23 pre-exposed neutrophils (Figure 6.7 E). These results suggest that IL-23 alone at relevant physiological concentrations does not promote neutrophil activation.

6.3.4 IL-23 exerts direct adverse effects on colonic epithelial cells by increasing intra-cellular ROS

Anti-IL-23 therapy in older *Winnie* mice with established colitis alleviated histopathology in the absence of any decrease in proinflammatory cytokine gene expression in the colon, indicating that IL-23 could promote inflammation via other cell types. In addition to downregulating *Mip2α* mRNA in colonic epithelial cells, anti-p19 antibody treatment also partially restored mucin production in the distal colonic goblet cells of *Winnie* mice (Figure 6.8 A, B). Alcian blue and PAS staining revealed that the relative volume of mature mucin stored in goblet cell theca within the crypt was increased, suggesting improved mucin biosynthesis in goblet cells of *Winnie* mice with anti-p19

treatment. Interestingly, mRNA levels of the major mucin in the mouse intestine, Muc2, were only slightly elevated with anti-p19 antibody treatment in *Winnie* mice (Figure 6.8 C), suggesting that post-transcriptional regulatory mechanisms or improved protein biosynthesis pathways could underlie increased mucin production by the intestinal goblet cells.

Given the improved goblet cell phenotype and decreased *Mip2α* gene expression in intestinal epithelial cells following anti-IL-23 treatment, and reports that colonic epithelial cells express the IL-23 receptor (Suzuki et al., 2012), the direct effects of IL-23 on human LS174T colon cancer cells which include MUC2-producing goblet cells were investigated. It has been shown previously that proinflammatory cytokines induce ER stress by increasing cellular oxidative stress (Xue et al., 2005), therefore ROS were measured by DCFDA oxidation after 2 h IL-23 treatment in LS174T cells. LS174T cells have been cultured in serum-free DMEM media for at least 12 h before the assay was performed, Intra-cellular ROS increased significantly in LS174T cells after exposure to IL-23 (Figure 6.9 A). Pharmacological inhibition studies showed that IL-23-induced ROS production was dependent on the STAT3 and STAT5 transcription factors known to be downstream of IL-23R signalling (Figure 6.9 A). Interestingly, ER stress (measured by the ERAI reporter for XBP1-splicing) was also substantially increased by IL-23 (Figure 6.9 B). IL-23-induced ER stress was dependent on STAT3 activation and production of ROS, but not STAT5 (Figure 6.9 B). Production of IL-8, the human chemokine equivalent to Mip-2α, by LS174T cells was significantly increased after exposure to IL-23 for 24 h (Figure 6.9 C). IL-23-induced IL-8 production was dependent on STAT3 and partially on ROS, but not STAT5 (Figure 6.9 C). Administering exogenous ROS at increased concentrations caused a dose-dependent increase in IL-8 secretion (Figure 6.9 D). STAT5 mediated IL-23 induced ROS production, but not ER stress or downstream chemokine release, suggesting that ER stress could be promoted independently of oxidative stress. Further study is needed to elucidate the detail of the intracellular pathways activated by IL-23 in colonic epithelial cells.

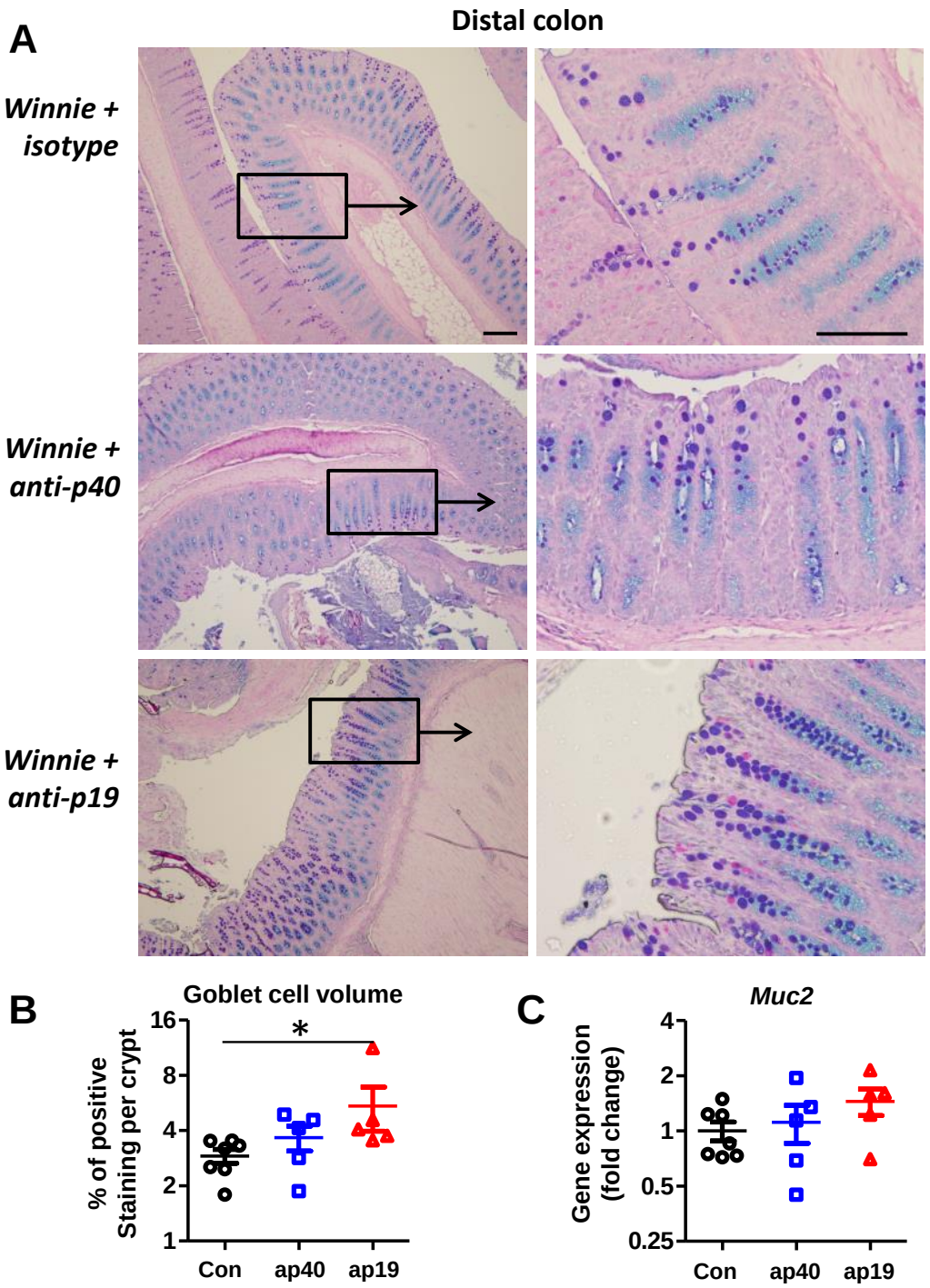


Figure 6.8 Two weeks treatment using anti-p19 antibody partially restored mucin production in colonic goblet cells of *Winnie* mice with established colitis. *Winnie* mice at 12 weeks of age were treated for 2 weeks with anti-p40 and anti-p19 antibodies. (A) AB/PAS staining showing goblet cell mucin stores in the distal colon. (B) Quantitation of AB/PAS staining in the distal colon. (C) *Muc2* gene expression in the distal colon relative to untreated control *Winnie* mice. Statistics: Mean ± SEM and individual data points, n=5-7, mixed gender, representative data from two experiments; B: One-way ANOVA with Bonferroni's multiple comparison test (*P< 0.05). Scale bars = 100 µm.

The secreted mucin, MUC2, contains N- and C-terminal cysteine-rich domains which require N-glycosylation in the ER, and formation of intra- and inter-molecular disulphide bonds, extensive folding and dimerization before transporting to the Golgi complex. Consequently, MUC2 biosynthesis is closely related to ER capacity and affected by ER stress (McGuckin et al., 2010). However, the effect of ROS on MUC2 production has not been studied. Thus, I hypothesized that IL-23 may suppress MUC2 production by inducing ER stress and increasing intracellular ROS. Total MUC2 protein in LS174T cells was significantly downregulated by 24 h treatment with IL-23 (Figure 6.9 E). IL-23-mediated inhibition of MUC2 production was blocked by the STAT3 inhibitor and ROS inhibitor (reduced glutathione), but not the STAT5 inhibitor (Figure 6.9 E). Exogenous ROS decreased MUC2 biosynthesis and secretion in a dose dependent manner with near complete inhibition above 10 μM (Figure 6.9 F). Overall, these results show that IL-23 directly induces ER stress via increasing ROS production within colonic epithelial cells in a STAT3-dependent manner, which in turn induces IL-8 secretion as well as suppressing mucin biosynthesis and secretion.

ROS have previously been shown in various cell types to induce IL-8 transcription and secretion via NFκB activation (p65 phosphorylation) (Gloire et al., 2006). I therefore hypothesized that IL-23-induced IL-8 secretion is dependent on NF-κB activation. To address this hypothesis, LS174T cells were treated with 50 ng/ml of IL-23. Activation of two major NF-κB pathway components, p65 phosphorylation and IκBα phosphorylation, were assessed over a time course by immunoblotting. Comparing to positive control TNF-α treatment, IL-23 treatment of LS174T cells did not promote p65 phosphorylation at serine 468 or IκBα phosphorylation (Figure 6.10 A), suggesting that IL-23 induced IL-8 production is not via the canonical NF-κB pathway. However, inhibition of IL-8 production by the NF-κB inhibitor BAY11-7082 (Figure 6.10 B) suggests that IL-23 could promote IL-8 secretion through the non-canonical NF-κB pathway. Interestingly, a basal level of p65 phosphorylation at serine 536 was detected in LS174T cells (Figure 6.10 A), but IL-23 had no effect on serine 536 phosphorylation. IL-23 stimulated p38 mitogen-activated protein kinase (MAPK) phosphorylation between 15 to 60 min later (Figure 6.10 A). The p38 MAPK phosphorylation inhibitor SB 203580 inhibited IL-8 production by IL-23 and the inhibitory effect was enhanced in combination with the NFκB inhibitor BAY11-7082 (Figure 6.10 B). In summary, the direct effects of IL-23 on colonic epithelial cells is via STAT3

dependent oxidative stress and ER stress induction, which further promotes IL-8 secretion through activation of the non-canonical NF-κB pathway and p38 MAP kinase (Figure 6.10 C).

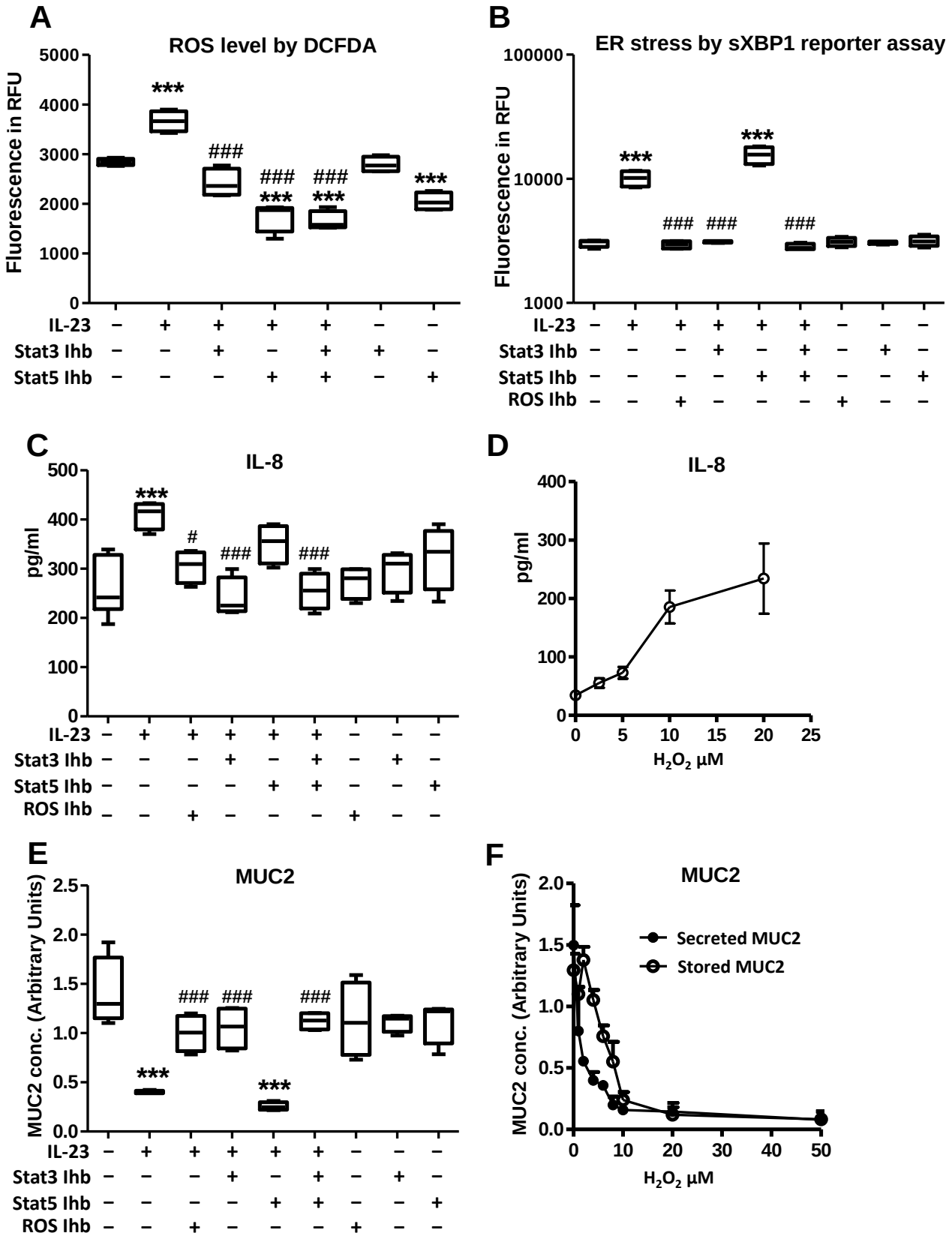


Figure 6.9 IL-23 directly stimulates proinflammatory chemokine production and suppresses mucin production by intestinal epithelial cells. (A) Intracellular ROS in LS174T cells ± 50 ng/mL IL-23 and/or 50 µM Stat3 inhibitor-VI S31-201 (Stat3 Ihb) or 50 µM Stat5 inhibitor CAS 285986-31-4 (Stat5 Ihb) for 2h. (B) ER stress indicated by XBP1 splicing (C) IL-8 production (E) Total MUC2 protein measured by ELISA in LS174T cells ± 50 ng/mL IL-23 and/or Stat3 Ihb, Stat5 Ihb or 50 µM ROS Ihb (reduced-glutathione) for 24h. (D) IL-8 production by LS174T cells exposed to 2.5-20 µM ROS (H_2O_2) for 4 h. (F) Intracellular and secreted MUC2 protein measured by ELISA in response to 24 h exposure to increasing concentrations of ROS. Statistics: A, B, C, D, E, F: Box plots showing median and inter-quartile range, n=4-6, experiment performed three times; One-way ANOVA with Bonferroni's multiple comparison test (#P< 0.05, */###P< 0.001). * Compared to the control group; # compared to the IL-23 treated group.**

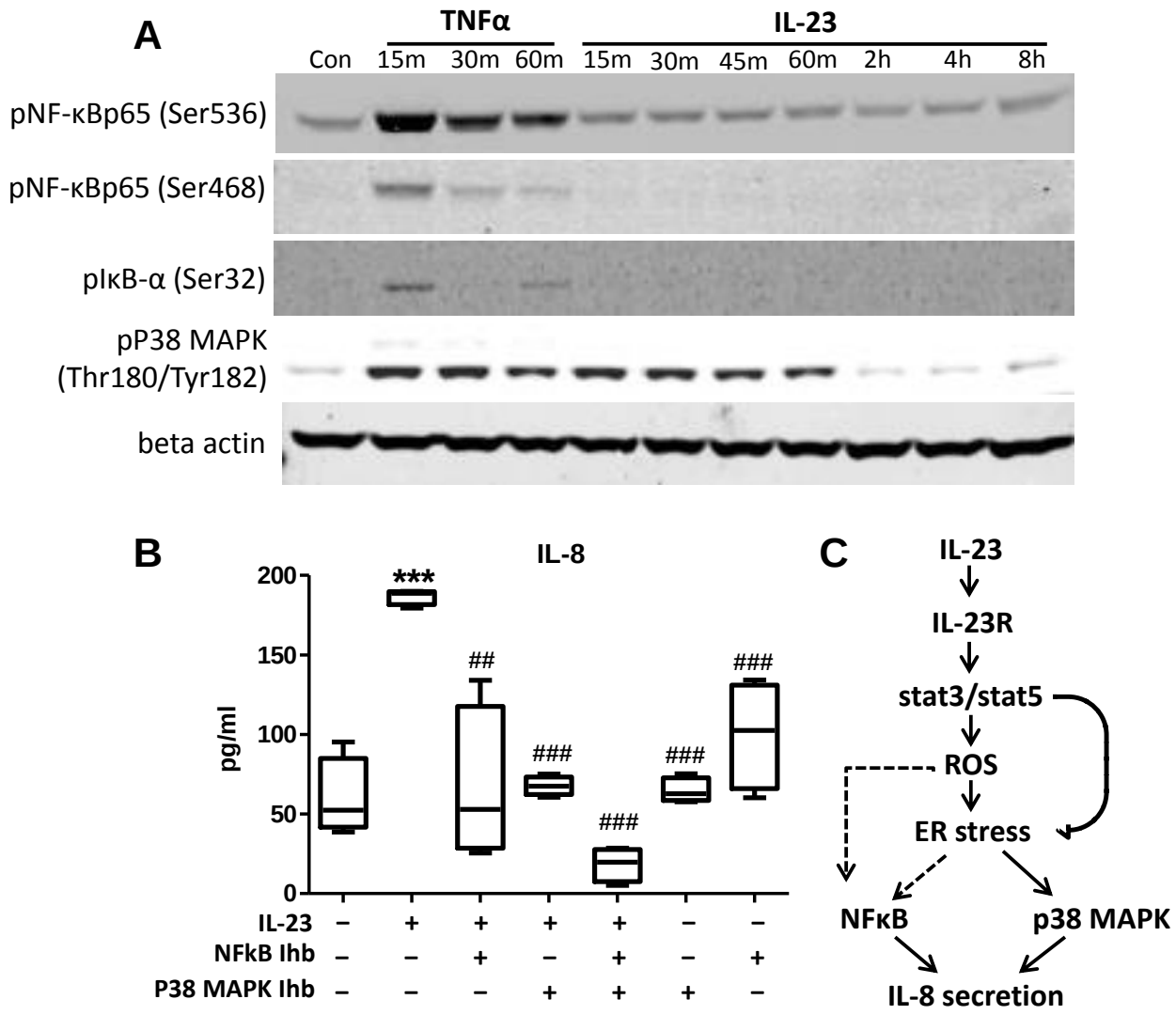


Figure 6.10 IL-23 drives proinflammatory chemokine IL-8 production in LS174T cells via activation of the NFκB and p38 MAPK pathways. (A) LS174T cells were treated with TNF-α (50 ng/ml) or IL-23 (50 ng/ml) for the indicated times. Whole cell lysate was subjected to SDS-PAGE electrophoresis and immunoblotted with indicated antibodies. (B) IL-8 production in LS174T cells ± 50 ng/mL IL-23, 20 μM NFκB Ihb (BAY11-7082) and 20 μM phospho-p38 MAPK Ihb (SB 203580) for 24 h. (C) Flow chart showing proposed mechanism of IL-23 induced IL-8 production in colonic epithelial cells. Statistics: B: Box plots showing median and inter-quartile range, n=4-6, experiment performed three times; One-way ANOVA with Bonferroni's multiple comparison test (##P< 0.01, */###P< 0.001). *Compared to the control group; # Compared to the IL-23 treated group.**

6.4. Discussion

The experiments reported in this chapter demonstrate that neutralization of IL-23 with an anti-p19 antibody suppressed emerging and established colitis in *Winnie* mice. The anti-p19 antibody was superior to anti-p40 antibody in suppressing intestinal inflammation. As predicted, anti-IL-23 treatment suppressed emerging colitis in the *Winnie* model, accompanied by restrained production of the proinflammatory cytokines, IL-1β and IL-17A. However, in mice with more advanced colitis, anti-p19 antibody treatment alleviated intestinal inflammation without suppressing IL-1β and IL-17A, and also partially restored the mucin production in intestinal goblet cells and reduced chemokine Mip-2α release by colonic epithelial cells.

Compared to mice treated with anti-p40 antibody, older *Winnie* mice with advanced colitis receiving anti-p19 antibody had elevated IFN-γ production from mesenteric lymph node leucocytes, suggesting that suppressing the IL-23/T_H17 axis promotes T_H1 cytokine production. Interestingly, this increased IFN-γ production was accompanied by decreased intestinal pathology. Aligned with this finding, it has been shown in an anti-CD40 colitis model that IL-12/T_H1 is responsible for acute systemic inflammation, but not local intestinal pathology. In this model IL-23 and the innate sources of IL-17A, such as NK cells and innate lymphoid cells, may be essential for local intestinal inflammation, but play no role in systemic responses (Uhlig et al., 2006).

Although anti-p40 antibodies have been shown to be effective in inducing remission and clinical response in patients with active CD in several clinical trials (Mannon et al., 2004; Sandborn et al., 2008; Sandborn et al., 2012), our results in this model suggest that in some forms of colitis, perhaps more likely in forms of ulcerative colitis, anti-p19 antibody may be more efficacious than anti-p40 antibody in suppressing intestinal inflammation. The effects of anti-p40 antibody in suppressing colitis needs further evaluation; particularly useful would be comparison with an anti-IL-12p35 antibody. These assessments of the efficacy of the anti-p19 antibody in the *Winnie* colitis model provide an interesting relevance for clinical therapy, as *Winnie* mice have a competent immune system and respond well to the anti-inflammatory drugs used clinically in IBD. Our results provide support for future clinical treatment strategies targeting IL-23 to suppress intestinal inflammation.

In *Winnie* mice with established colitis, anti-IL-23 treatment significantly reduced neutrophil infiltration which was not observed with either anti-IL-17A or anti-IL-17Ra treatment (see 5.3.1 to 5.3.3), indicating that IL-23 directly or indirectly mediates neutrophil recruitment independently of IL-17A. Supporting this hypothesis, other studies have shown that IL-23-dependent neutrophil recruitment is protective for colonic epithelial cells in the resolution stage of DSS colitis (Zindl et al., 2013). However, results from this chapter show that 50 ng/ml IL-23 treatment or pre-exposure did not modify the activation state of neutrophils as assessed by intra-cellular ROS. The discrepancy with a previous study (Taylor et al., 2014) could be due to pre-activation of neutrophils during the isolation and preparation process. A recent study has demonstrated that IL-23 and IL-6 are directly involved in neutrophil activation and recruitment during fungal infection (Taylor et al., 2014). In this study, cytokine stimulated (IL-6 at 20 µg/ml and IL-23 at 2 µg/ml) neutrophils had higher intracellular ROS compared to unstimulated neutrophils (Taylor et al., 2014). However, I was unable to replicate these results, perhaps because I used lower concentrations of cytokines than the extremely high concentration of cytokines used in the previous study. Interestingly, another study revealing a novel mechanism of neutrophil activation shows that neutrophils are a major cell source for IL-1β during acute infection via the NLRC4 inflammasome, despite the fact that they are also a cellular target of IL-1β (Chen et al., 2014). It is possible that IL-23-dependent colonic neutrophil infiltration is sustained and amplified through an autocrine loop of neutrophil-derived IL-1β together with colonic epithelial cell inflammasome activation. Blocking IL-23 may alleviate local neutrophil infiltration as well as suppressing IL-1β-sustained local neutrophil activation.

Prior to this study, the effects of IL-23 on non-lymphoid cells and IL-23 receptor expression on epithelial cells have only been reported in human lung cancer cells (Li et al., 2013) and colon cancer cells (Suzuki et al., 2012). Results in this chapter show that IL-23 has direct effects on intestinal epithelial cells by STAT3 and STAT5-dependent induction of ROS, which in turn induces ER stress. Increased oxidative stress and ER stress further stimulate proinflammatory chemokine production and reduces MUC2 production, the major component of the intestinal mucus which separates the microbiota from the epithelium. However, I used LS174T colon cancer cells and it is possible that cancer cells express higher level of IL-23R than normal epithelial cells. IL-23R expression in intestinal epithelial cells in health and during inflammation requires careful evaluation.

NF-κB mediated IL-8 secretion has been extensively studied in various cell types and inflammation models. Two major pathways mediate NF-κB activation, namely the canonical and non-canonical pathways. Canonical pathway activation is mediated by inducible degradation of IκBα via its phosphorylation by the IκB kinase (IKK). IκBα phosphorylation is associated with ubiquitination and dissociation from NFκB (p65/p50), which leads to the translocation of the activated transcription factor into the nucleus and induction of gene expression (Oeckinghaus and Ghosh, 2009). The non-canonical pathway activates other NF-κB complexes independent of IκBα degradation (Sun, 2012). Considering the fact that BAY 11-7082 is an IKK inhibitor with poor selectivity for IKKα and IKKβ (Rauert-Wunderlich et al., 2013) and IKKα (not IKKβ or IKKγ) is required for non-canonical NF-κB signalling (Claudio et al., 2002; Hacker and Karin, 2006), it is possible that IL-23-induced IL-8 production is via non-canonical NF-κB activation. The basal level of p65 phosphorylation at serine 536 in LS174T cells could also due to activation of the non-canonical pathway, as p65 specific serine 536 phosphorylation has been shown to be independent of canonical NF-κB activation (Rauert-Wunderlich et al., 2013). Therefore IL-23-stimulated chemokine production could be dependent on both oxidative and ER stress-triggered MAPK activation and non-canonical NF-κB activation by other inflammatory or cell-intrinsic factors.

In summary, due to IL-23's multiple mechanisms of action, neutralization of IL-23 in colitis is likely to prove efficacious by reducing T cell and ILC activation, reducing chemokine-mediated recruitment of inflammatory cells into the intestine, and by replenishing intestinal mucus, thereby helping restore the normal homeostatic state. The previously unrecognized action of IL-23 on epithelial cells also suggests that the protection from IBD conveyed by hypomorphic IL-23R alleles may be at least partially explained by reduced adverse effects of IL-23 on intestinal epithelial cells.

7.0 Discussion

The intestinal epithelium is a critical regulator of the intestinal immune system, and the cross talk between the intestinal epithelium and underlying immune system is likely to be crucial in IBD pathogenesis. Currently there is enormous interest from researchers and the pharmaceutical industry in targeting proinflammatory immune responses, including the T_H17 response, to treat IBD. Anti-cytokine monoclonal antibodies are a promising form of biological therapy, with anti-TNF-α antibodies in routine use to induce remission in CD and, to a lesser extent, UC patients. Given anti-IL-17A antibodies show substantial efficacy in treating autoimmune diseases like plaque psoriasis (Blauvelt et al., 2014) and ankylosing spondylitis (Baeten et al., 2013), secukinumab, an anti-IL-17A monoclonal antibody has been trialed for treatment of moderate to severe Crohn's disease. Unfortunately, secukinumab was ineffective and increased adverse events were reported compared with placebo (Hueber et al., 2012). Therefore, the specific role of the T_H17 immune response in intestinal inflammation needs further examination, as a number of different interacting cytokines and complex cross-talk between epithelial cells and leukocytes are involved. This project aimed to investigate the physiological role of the IL-17A - IL-17Ra pathway in intestinal inflammation and to assess the efficacy of monoclonal antibodies against T_H17 cytokines, including IL-23, in suppressing intestinal inflammation utilizing a spontaneous chronic colitis model in mice.

The major findings from this project are summarized as follows:

- After transfer of naïve T cells, lymphopenic *RaW* recipients with an epithelial defect had more severe intestinal inflammation compared to *Rag1*$^{-/-}$ recipients. Although IL-17A deficiency in T cells had no effects on transfer colitis development in recipient mice, transfer of IL-17Ra-deficient T cells exacerbated colitis compared to WT T cells, suggesting that the IL-17A – IL-17Ra pathway in does not promote this form of colitis and in fact may repress T cell-mediated pathophysiology.

- Systemic deficiency of IL-17A suppressed the age-related progression of colitis in *Winnie* mice. However, neutralizing IL-17A, IL-17Ra or other T_H17 associated cytokines using monoclonal antibodies failed to suppress *Winnie* colitis. Thus,

the therapeutic efficacy of targeting the T_H17 pathway alone with antibodies is minimal.

- Neutralizing IL-23 using anti-p40 and anti-p19 antibodies suppressed emerging and established colitis in *Winnie* mice and anti-p19 was more efficacious than anti-p40 antibody. Based on previous knowledge the efficacy of anti-IL-23 is attributable to its multiple effects on immune cells including recruiting neutrophils, promoting the maturation of inflammatory T_H17 cells and ILCs, and suppressing the activity of regulatory T cells (Figure 7.1).

- In a novel finding, IL-23 drove intra-cellular ROS production in intestinal epithelial cells via STAT3 and STAT5, which further promoted ER stress. Increased ROS concentrations and subsequent ER stress inhibited MUC2 biosynthesis and stimulated secretion of the chemokine, IL-8, via NF-κB and p38 MAPK activation. Thus it is likely that the efficacy of anti-IL-23 in suppressing colitis is also due to the previous unrecognized effects of IL-23 on intestinal epithelial cells (Figure 7.1).

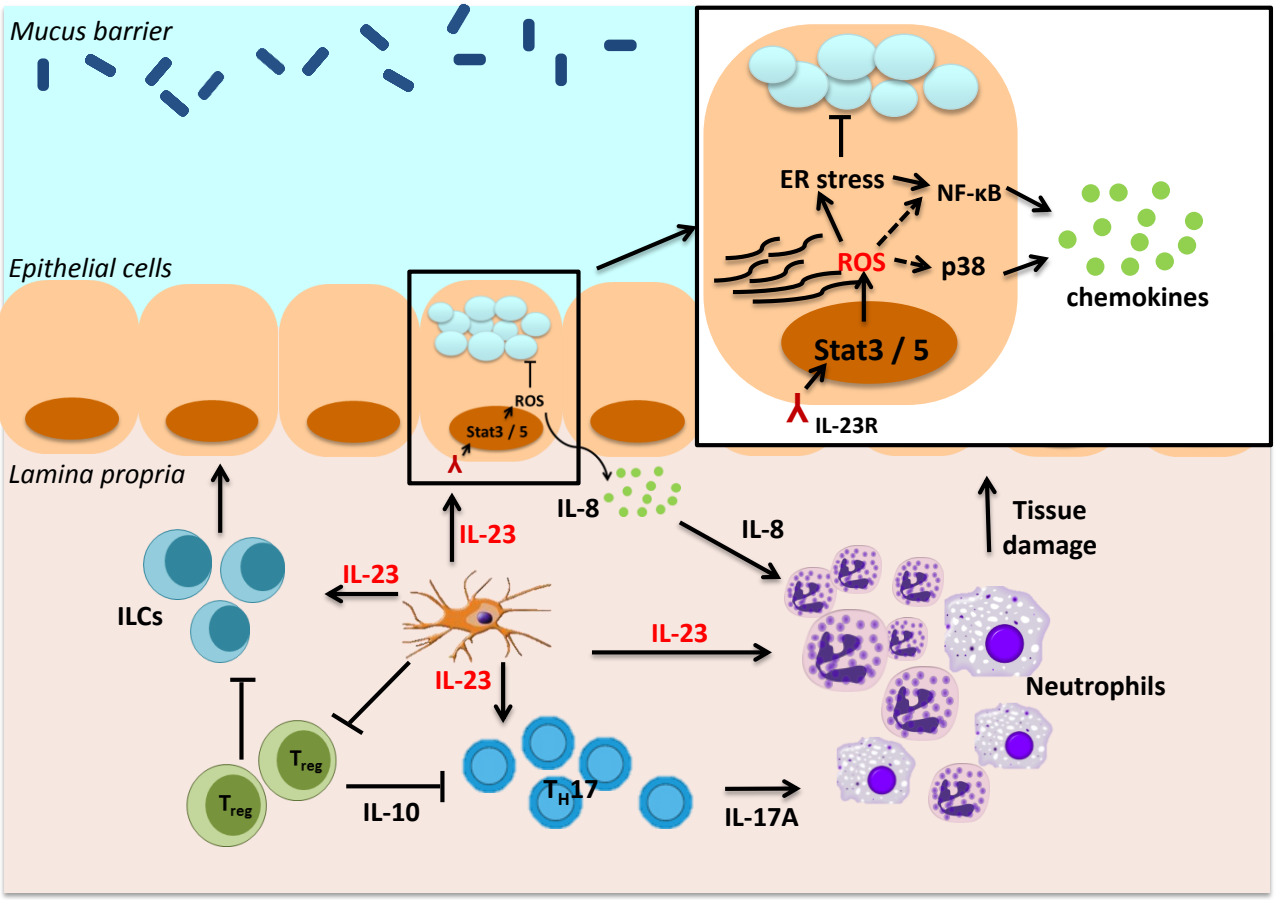


Figure 7.1 IL-23 has multiple effects on immune and non-immune cells that drive intestinal inflammation. IL-23 produced by antigen presenting cells drives inflammatory T_H17 cell and ILC maturation and cytokine production. IL-23 could directly recruit or activate neutrophils via an IL-17A-independent mechanism and also via IL-17. IL-23 suppresses regulatory T cell function which further promotes expansion of inflammatory T cells and ILCs. Besides the effects on immune cells, IL-23 acts directly on colonic epithelial cells by STAT3 and STAT5-dependent induction of ROS, which in turn induces ER stress. Increased oxidative stress and ER stress stimulates release of the IL-8 chemokine and reduces MUC2 production, the major component of the intestinal mucus which separates the microbiota from the epithelium. IL-23 induced IL-8 production in epithelial cells is dependent on NF-κB and p38 activation, and this chemokine further contributes to local neutrophil recruitment and tissue damage. IL-23 is a major contributor to local intestinal inflammation mediated by diverse adverse effects on immune and non-immune cells as colitis progress.

In contrast to the findings of O'Connor et al. showing that *IL-17A*$^{-/-}$ T cells are more colitogenic than wild type T cells in driving transfer colitis (O'Connor Jr et al., 2009), we found that IL-17A deficiency did not modify the colitogenic ability of T cells. However, our finding is supported by three other studies (Izcue et al., 2008; Leppkes et al., 2009; Noguchi et al., 2007). Although we concur with O'Connor's findings that *IL-17Ra*$^{-/-}$ T cells generate more severe colitis than wild type T cells, we could not find evidence of skewing to a T_H1 immune response, which is supported by similar finding from Izcue et al. (Izcue et al., 2008). Reasons for the discrepancy between results is unknown, but differences in the microbiota between facilities may partly explain the divergence, as transfer colitis development is intestinal microbiota dependent (Feng et al., 2010). The difference observed between *IL-17A*$^{-/-}$ T cell and *IL-17Ra*$^{-/-}$ T cell transfer experiments could be due to the unresponsiveness of *IL-17Ra*$^{-/-}$ T cells to other cytokines like IL-17F or IL-17E (Iwakura et al., 2011). The transfer colitis model is a useful tool to investigate the role of T cell-mediated inflammatory responses in colitis development, but it has limitations in translating findings to clinical therapy. IBD patients are not lymphopenic, and multiple subsets of immune cells contribute to the immune pathogenesis including innate lymphoid cells, γδT cells and regulatory T cells.

In contrast to the results of IL-17A deficiency in T cells, systemic deficiency of IL-17A alleviated colitis progression in *Winnie* mice, suggesting that non T cell-derived IL-17A is involved in colitis progression. Previous findings from our group show that *Il17a* gene expression in the intestinal mucosa of *Winnie* mice is significantly upregulated while aging (Eri et al., 2011), suggesting that IL-17A contributes more to the progression than the initiation stage of colitis. It is possible that immune cell-derived IL-17A has different effects in different stages as colitis progresses. An interesting finding from another study shows that naïve T cells from aged C57BL/6 mice have dramatically elevated *Il17a* gene expression compared to T cells from young mice, which resulted in more severe transfer colitis in *Rag1*$^{-/-}$ recipients compared to cells from young mice (Ouyang et al., 2011). Based on these findings, it is likely that potentiation of IL-17A in the aging process contributes to the development of chronic colitis. However, impacts of genetic deficiency of IL-17A on chronic colitis development have not been comprehensively studied in general. A previous study showed that *IL-17A*$^{-/-}$ mice had exacerbated DSS-colitis compared to wild type mice (Yang et al., 2008a), suggesting a regulatory effect of IL-17A in this acute model of intestinal inflammation. It is possible that IL-17A plays different roles in chronic and

acute intestinal inflammation. It's been reported that IL-17A is able to promote claudin expression and regulate intestinal epithelial tight junctions (Kinugasa et al., 2000).

Although IL-17A contributes to chronic colitis progression, targeting IL-17A or IL-17Ra failed to show efficacy in the *Winnie* colitis model. A recent study shows that anti-IL-17A antibody alone failed to suppress naïve T cell induced transfer colitis (Wedebye Schmidt et al., 2013). In an IL-10-deficiency induced colitis model, blockade of IL-17A by monoclonal antibody also lacked efficacy unless IL-6 was also neutralized (Yen et al., 2006). Similar disappointing results were reported in human trials. Two different anti-IL-17A antibodies failed to show efficacy in treating active CD patients (Hueber et al., 2012; Targan et al., 2012). Increased fungal infections (Hueber et al., 2012) and increased disease severity (Targan et al., 2012) were reported. These results are not entirely surprising, considering the evidence that colonization by the commensal flora drives T_H17 cell development (Chung et al., 2012; Ivanov et al., 2009; Yang et al., 2014) and the physiological function of T_H17 cells is to fight against extra-cellular pathogens at mucosal sites (Ivanov et al., 2009; Yang et al., 2014). In the *Winnie* spontaneous colitis model induced by a defect in the secretory epithelial cells that produce the mucus barrier, the protective aspects of T_H17 immunity dealing with microbes that penetrate the defective barrier is probably as important as restraining the T_H17 response to alleviate associated tissue damage. Within this complex inflammatory environment, we found that taking out single effector cytokines or blocking specific cytokine receptors was insufficient to fine tune the balance and suppress local inflammation, with the exception of neutralization of IL-23. The contrast with the constitutive loss of IL-17 from birth in *IL-17*$^{-/-}$*Winnie* mice may relate to different evolution of mucosal immunity in the complete absence of IL-17, and therefore not reflect the clinical situation in IBD.

Since IL-17A negatively regulates IFN-γ-producing T_H1 cells (O'Connor Jr et al., 2009) and increased IFN-γ production was observed in *Winnie* mice receiving anti-IL-17A antibody (see 5.3.1 for details), a more rational approach to suppress intestinal inflammation may be to simultaneously target the T_H1 and T_H17 pathways. The efficacy of neutralizing IFN-γ and IL-17A together using monoclonal antibodies is worth exploring. Another possible cytokine to target is IL-21, as IL-21 sustains both T_H1 and T_H17 cytokine production in cells isolated from the mucosa of IBD patients (Fina et al., 2008; Monteleone et al., 2005b). Furthermore, IL-21-deficient mice were protected against DSS and TNBS

colitis and were unable to upregulate T_H1 and T_H17 proinflammatory cytokines during intestinal inflammation (Fina et al., 2008). Administration of an IL-21 receptor fusion protein suppressed inflammatory cytokine production and DSS colitis in wild type mice (Fina et al., 2008). In the future, it would also be interesting to explore the efficacy of anti-IL-17A antibodies as maintenance therapy after induction of remission by steroids or 5-ASA in the *Winnie* model which responds well to these agents. However, long-term use of anti-IL-17A could also potentially increase the risk of opportunistic infections in this setting. In summary, targeting the major T_H17 effector cytokine, IL-17A, appears in multiple murine models and human trials to have little efficacy in suppressing colitis.

IL-23 plays a crucial role in colitis development due to its effects on multiple immune cells including T_H17 cells (Stritesky et al., 2008; Yen et al., 2006), ILCs (Eken et al., 2014; Uhlig et al., 2006) and T_{reg} cells (Izcue et al., 2008; Liu et al., 2012). GWAS reveal that *IL23R* gene polymorphisms are strongly associated with IBD susceptibility (Jostins et al., 2012a). The most common polymorphism appears to be a protective hypomorphic allele (Duerr et al., 2006b), suggesting that IL-23 signalling is involved in the development of disease. To date, blocking IL-23 using monoclonal antibody therapy is still under clinical investigation and its potential to suppress intestinal inflammation is yet to be fully characterized. Murine studies have demonstrated significant efficacy of anti-IL-23 antibody in treating inflammatory or autoimmune diseases, including arthritis (Yago et al., 2007), spondyloarthritis (Benham et al., 2014) and autoimmune encephalomyelitis (Chen et al., 2006). In a recent clinical trial in moderate-to-severe psoriasis, more patients treated with guselkumab (anti-IL-23 antibody) had clinical responses compared to a placebo control group (Sofen et al., 2014). The efficacy of anti-IL-23 therapy has been associated with suppression of IL-17-dependent inflammatory responses. Targeting IL-23 shows promising efficacy in treating inflammatory or T_H17-mediated autoimmune diseases. In targeting IL-23, antibodies against p40, a shared subunit of IL-12/23 cytokines, have been trialed in treating active CD. Two fully humanised IgG1 monoclonal antibodies against p40 subunit have been developed: briakinumab and ustekinumab. Although ustekinumab is effective as induction and maintenance therapy in treating subsets of CD patients who had previously failed anti-TNF therapy (Sandborn et al., 2012), the overall efficacy of targeting the p40 subunit in suppressing intestinal inflammation is not impressive (Mannon et al., 2004; Sandborn et al., 2008). To date, these antibodies haven't been tested or trialed in UC yet.

One of the most important and unexpected findings of this project was that IL-23 acts directly on colonic epithelial cells. This result not only defines IL-23 as a key cytokine driving intestinal inflammation, but also adds to a growing recognition that epithelial cells are actively involved in regulating local immune responses and maintaining homeostasis (Kagnoff, 2014; Kaser et al., 2011; McGuckin et al., 2009; McGuckin et al., 2010; Peterson and Artis, 2014). Early evidence demonstrates that intestinal epithelial cells can respond to luminal microbial signals and condition antigen presenting cells and subsequently lymphocytes to elicit immune function (Rimoldi et al., 2005). With increasing evidence, it is now clear that epithelial cells can directly initiate and then promote inflammatory responses such as those associated with IBD pathogenesis. Multiple murine models with alterations in epithelial barrier function develop spontaneous intestinal inflammation with phenotypes similar to human IBD (Heazlewood et al., 2008; Kosiewicz et al., 2001; Nenci et al., 2007; Van der Sluis et al., 2006). We have shown here that in response to IL-23 epithelial cells develop oxidative stress and ER stress which in turn can promote local intestinal inflammation by secreting proinflammatory chemokines. It is likely that this pathway has evolved so that APC-derived IL-23 can help protect epithelial cells from intracellular pathogens via the production of ROS and to amplify leukocyte recruitment by the epithelial cell chemokine production. This finding provides further evidence of the importance of oxidative stress and ER stress in colonic epithelial cells as both a primary cause of intestinal inflammation or a consequence of inflammation (Adolph et al., 2013; Hasnain et al., 2013; Kaser et al., 2011).

We have shown evidence that IL-23 acts directly on colonic epithelial cells, but LS174T cells are malignant cancer cells which may not represent the real physiological scenario. Prior to our study, IL-23 receptor expression on epithelial cells has only been reported in human lung cancer cells (Li et al., 2013) and colon cancer cells (Suzuki et al., 2012). Therefore, it would be interesting and necessary to examine the IL-23R expression level *in vivo* and the physiological role of IL-23R signaling in primary colonic epithelial cells in the future. To address this, intestinal epithelial cell conditional IL-23R knockout mice would be a useful tool. In fact supporting our findings, a very recent conference report using mice with conditional knockout of the *Il23r* in the intestinal epithelium (IL23RIEC-KO) shows that IL23RIEC-KO mice produce less antimicrobial peptides, have a disturbed colonic microflora and succumb to DSS colitis (Aden et al., 2014). Another approach to

study the physiological role of IL-23R in epithelial cells would be to generate bone marrow chimeras. For example, it would be interesting to compare the chemical toxin-induced intestinal inflammation between IL-23R-deficient and wild type mice reconstituted with wild type vs IL-23R-deficient bone marrow.

Another interesting area needing further study is the effect of IL-23 on colonic epithelial cell IL-1β production and downstream neutrophil infiltration. Recently, an interesting study revealed a novel mechanism of neutrophil activation and showed that neutrophils are a major cell source for IL-1β during acute infection via the NLRC4 inflammasome, despite the fact that they are also a cellular target of IL-1β (Chen et al., 2014). It is possible that IL-23-dependent colonic neutrophil infiltration is sustained and amplified through an autocrine loop of neutrophil-derived IL-1β together with colonic epithelial cell inflammasome activation. Blocking IL-23 may alleviate local neutrophil infiltration as well as suppressing IL-1β-sustained local neutrophil activation. Direct inflammasome activation by IL-23 has not been reported before. However, it has been shown that ER stress can induce IL-1β secretion in macrophages via NLRP3 inflammasome activation, and both potassium efflux and increased ROS levels are required for this process (Menu et al., 2012; Oslowski et al., 2012). Therefore, it is reasonable to hypothesize that IL-23 induces IL-1β production via inflammasome activation in colonic epithelial cells given the fact that IL-23 is a strong inducer of oxidative stress and ER stress.

Suppressing inflammation, maintaining remission and limiting side effects are the ultimate goals for treating IBD in the clinic. Consequently, biological therapies targeting specific proinflammatory cytokines to suppress intestinal inflammation are under extensive study. The major concept of this project was to identify potential therapeutic targets by using neutralizing monoclonal antibodies against T_H17-associated proinflammatory cytokines. Disappointing results have demonstrated that neutralizing antibodies against IL-17A and IL-17Ra failed to show any efficacy. One of the frequently raised questions regarding this negative result is how to evaluate the neutralizing efficiency of these antibodies *in vivo*. We were unable to assess this from our animal experiments; however, these same antibodies and doses have been used successfully to suppress immunopathology in other murine models of inflammatory disease. To address the question of efficacy of neutralization in the future, it would be necessary to investigate the

antibody binding efficiency *in vivo* through pharmacological studies including monitoring the systemic concentration as well as the local mucosa concentration of the antibody and both free and bound cytokine, which is technically challenging.

This project using a unique mouse model of intestinal inflammation provided an opportunity to investigate the role of stressed epithelial cells in regulating DC and other immune responses. Unlike other murine experimental models where colitis is induced by exogenous chemical toxins or defects in the immune system, *Winnie* colitis arises from epithelial secretory cell ER stress and mucosal barrier defects which develop with an intact underlying immune system (Eri et al., 2011; Heazlewood et al., 2008). Anti-inflammatory drugs which are efficacious in treating human UC can also successfully suppress *Winnie* colitis (Das et al., 2013). This makes *Winnie* mice a useful murine model to screen potential therapeutic targets and to evaluate the efficacy of new biological therapies. Although the epithelial defect driven colitis in *Winnie* mice shares phenotypic similarities with human UC, the mucin misfolding and goblet cell ER stress leading to inflammation in *Winnie* mice cannot represent the overall disparate and complex disease pathogenesis in human UC. Differences between the physiology of the mouse and human intestine, including the distribution and lifespan of goblet cells in the mouse colon, could influence the translation of our findings to the treatment of human colitis.

In this project, I have shown that blocking IL-23 using an anti-p19 antibody is more efficacious than an anti-p40 antibody in suppressing *Winnie* colitis. The different efficacy of these two antibodies suggests that IL-23, not IL-12, is the major contributor to pathology in *Winnie* intestinal inflammation. However, to further dissect the difference, an anti-IL-12p35 antibody needs to be included to further evaluate the effects of anti-p40 antibody in suppressing colitis.

To date, there is no comprehensive study directly comparing the efficacy of anti-IL-17A and anti-IL-23 antibodies in experimental colitis models. I have shown that blocking T_H17 effector responses in colitis by targeting single effector cytokines or cytokine receptors was ineffective. However, neutralizing IL-23 not only suppressed leukocytes, but limited neutrophil infiltration into the intestinal mucosa and alleviated epithelial cell oxidative stress and ER stress. These findings deepen our understanding of the IL-

23/T_H17 axis and may help guide therapeutic approaches for patients with active inflammatory bowel disease.

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Appendix A

THE UNIVERSITY OF QUEENSLAND

UQ Research and Innovation
Director, Research Management Office
Nicole Thompson

ANIMAL ETHICS APPROVAL CERTIFICATE

30-Apr-2014

Activity Details

Chief Investigator:	Professor Michael McGuckin
Title:	Suppressing ER stress in IBD
AEC Approval Number:	MMRI/205/12/NHMRC
Previous AEC Number:	MMRI/957/08/UQ/MMRI/NHMRC
Approval Duration:	30-Aug-2012 to 30-Aug-2015
Funding Body:	MMRI, NHMRC, UQ
Group:	Health Sciences
Other Staff/Students:	Timothy Florin, Lisa Craig, Tony Kenna, Ran Wang, Wendy Tong, Sumaira Hasnain, Ann Burns-Hutchison
Location(s):	Other Queensland Location Other Victorian Location PA Hospital Translational Research Institute (TRI)

Summary

Subspecies	Strain	Class	Gender	Source	Approved	Remaining
Mice - genetically modified	Winnie	Adults	Mix	Institutional Breeding Colony	604	555
Mice - genetically modified	Xbp1-GFP	Adults	Mix	Institutional Breeding Colony	40	40
Mice - genetically modified	RAG1 -/-	Adults	Mix	Institutional Breeding Colony	448	448
Mice - genetically modified	IL-23R -/- (C57BL/6)	Adults	Mix	Institutional Breeding Colony	80	80
Mice - genetically modified	IL-23R +/- GFP (C57BL/6)	Adults	Mix	Institutional Breeding Colony	80	80
Mice - genetically modified	IL-23R +/+ (C57BL/6)	Adults	Mix	Institutional Breeding Colony	8	8
Mice - Inbred	C57BL/6	Adults	Mix	Institutional Breeding Colony	600	548
Mice - Inbred	Winnie x Rag1 -/- *	Adults	Mix	Institutional Breeding Colony	224	224
Mice - Inbred	Win/Win IL-17-/- *	Adults	Mix	Institutional Breeding Colony	62	30
Mice - Inbred	Winnie x Xbp1-GFP	Adults	Mix	Institutional Breeding Colony	40	40

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Permit(s):

Proviso(s):

Approval Details

Description	Amount	Balance
Mice - genetically modified (IL-23R -/- (C57BL/6), Mix, Adults, Institutional Breeding Colony)		
5 Jul 2013 Modification #3	80	80
Mice - genetically modified (IL-23R +/- GFP (C57BL/6), Mix, Adults, Institutional Breeding Colony)		
5 Jul 2013 Modification #3	80	80
Mice - genetically modified (IL-23R +/+ (C57BL/6), Mix, Adults, Institutional Breeding Colony)		
5 Jul 2013 Modification #3	8	8
Mice - genetically modified (RAG1 -/-, Mix, Adults, Institutional Breeding Colony)		
19 Jul 2012 Application	448	448
31 Dec 2012 Use in 2012 (from 2013 MAR)	0	448
Mice - genetically modified (Winnie, Mix, Adults, Institutional Breeding Colony)		
19 Jul 2012 Application	504	504
22 Nov 2012 Modfication #1	60	564
31 Dec 2012 Use in 2012 (from 2013 MAR)	-49	515
17 Apr 2014 Modification #6	40	555
Mice - genetically modified (Xbp1-GFP, Mix, Adults, Institutional Breeding Colony)		
19 Jul 2012 Application	40	40
31 Dec 2012 Use in 2012 (from 2013 MAR)	0	40
Mice - Inbred (C57BL/6, Mix, Adults, Institutional Breeding Colony)		
19 Jul 2012 Application	560	560
31 Dec 2012 Use in 2012 (from 2013 MAR)	-52	508
17 Apr 2014 Modification #6	40	548
Mice - Inbred (Win/Win IL-17-/-*, Mix, Adults, Institutional Breeding Colony)		
19 Jul 2012 Application	32	32
31 Dec 2012 Use in 2012 (from 2013 MAR)	-102	-70
1 Jan 2013 Administrative adjustment to zero on 2012 MAR	70	0
5 Jul 2013 Modification #2	30	30
Mice - Inbred (Winnie x Rag1 -/-*, Mix, Adults, Institutional Breeding Colony)		
19 Jul 2012 Application	224	224
31 Dec 2012 Use in 2012 (from 2013 MAR)	0	224
Mice - Inbred (Winnie x Xbp1-GFP, Mix, Adults, Institutional Breeding Colony)		
19 Jul 2012 Application	40	40
31 Dec 2012 Use in 2012 (from 2013 MAR)	0	40

Appendix B

Score Sheet for Intestinal Pathology Assessment in Mouse ♀/♂

Experiment number:_____

Mouse number:_____

Date of challenge:_____Drug (if any):___Dose: ____Pre-challenge weight (g):____

DATE				
Time post-challenge				
Observations from a distance				
Inactive				
Hunched posture				
Ruffled fur				
Rate of breathing				
Crusty eyes				
Shivering				
Diarrhea				
Rectal bleeding				
Rectal prolapse				
On handling				
Not inquisitive or alert				
Bodyweight (% change from start/score)*				
Any other abnormal behavior or signs noted				
Action taken				
Notes				
Total score				

* Scoring for bodyweight change (% change from start)

0 = <5% weight loss, 1 = 5-15% weight loss, 2 = 15-20%, 3 = >20% weight loss

Mice need to be sacrificed once the total score reach 3 or is more than 3.

Symptom	Mild Symptoms = 1	Severe Symptoms Score = 3
Inactive	Fully mobile, but more inactive than usual	Mouse moving only occasionally and largely unresponsive to prompting
Hunched posture	Small change in normal posture	Mouse noticeably hunched over without regaining normal posture
Ruffled fur	Some restricted ruffling of fur	Obvious ruffling of most fur
Rate of breathing	Slightly above or below normal	Excessive panting or markedly diminished rate of breathing
Crusty Eyes Shivering	Small buildup of excretion in eyes Occasional shivering	Significant excretion in both eyes, vision likely to be impaired Constant prominent shivering
Diarrhea	Loose stools	liquid consistency
Rectal bleeding	Some blood in stool on close inspection	Clear bleeding from rectum
Rectal prolapse	Rectum threatening to prolapse on defecation	Permanent prolapse
Not inquisitive or alert	Some reduced response to stimuli	Mouse largely unresponsive to stimuli

Appendix C

Score Sheets for Chronic/Acute Colitis

Animal ID

Crypt Architecture	0 = normal 1 = irregular 2 = moderate crypt loss (10-50%) 3 = severe crypt loss (50-90%) 4 = small/medium sized ulcers (<10 crypt widths) 5 = large ulcers (>10 crypt widths)
Crypt Abscesses	0 = none 1 = 1-5 2 = 6-10 3 = >10
Crypt Length (µm)	Caecum – 0 = < 130, 1 = 130-150, 2 = 150-200, 3 = 200-250, 4 = >250 PC – 0 = < 150, 1 = 150-200, 2 = 200-250, 3 = 250-300, 4 = >300 MC – 0 = < 250, 1 = 250-300, 2 = 300-400, 3 = 350-400, 4 = >400 DC – 0 = < 200, 1 = 200-250, 2 = 250-300, 3 = 300-350, 4 = >350
Tissue Damage	0 = no damage 1 = discrete lesions 2 = mucosal erosions 3 = extensive mucosal damage
Goblet Cell Loss	0 = normal <10% loss 1 = 10-25% 2 = 25-50% 3 = >50%
Inflammatory Cell Infiltration	0 = occasional infiltration 1 = increasing leukocytes in lamina propria 2= confluence of leukocytes extending to submucosa 3 = transmural extension of inflammatory infiltrates
Lamina Propria Neutrophils (PMN)	0 = 0-5 PMNs/HPF 1 = 6-10 2 = 11-20 3 = >20